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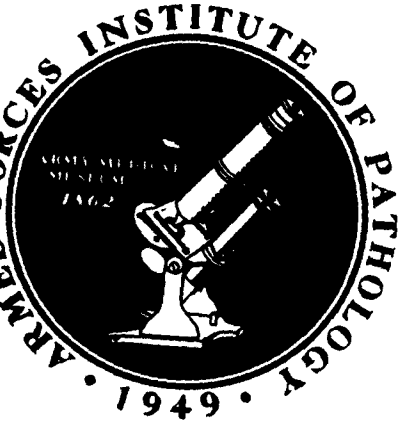

ANNUAL PROGRESS REPORT

For

Army Research and Development Command

Research Contracts

REPORTS CONTROL SYMBOL:

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1 July 1962 - 30 June 1963

ARMED FORCES INSTITUTE OF PATHOLOGY

Washington 25, D.C.

ANNUAL PROGRESS REPORT

REPORTS CONTROL SYMBOL: RCS-MEDDH-288

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ANNUAL PROGRESS REPORT

Project No. 3A012501B813

Task No. 1 Title Biochemistry: Correlation of Morphologic and Chemical Changes in Cells Exposed to Toxic Chemicals

Name & Address of Reporting Installation:

Armed Forces Institute of Pathology

Washington 25, D. C.

Name of Department(s) and Division(s):

Forensic Pathology Branch

Period Covered by the Report: 1 July 1962 - 30 June 1963

Professional Authors of the Report:

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Assistant Edward H. Johnston, Lt. Ccl, MC, USA

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ABSTRACT

Project No. 3A012501B813 Title Army Med. Basic Research in Life Sciences

Task No. 1 Title Biochemistry: Correlation of Morphologic and Chemical Changes in Cells Exposed to Toxic Chemicals

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology

Washington 25, D. C.

Authors:

Joseph S. Amenta, Capt, MC, USA

Edward H. Johnston, Lt. Col., MC, USA

Reports Control Symbol: RCS MEDDH-288

Security Classification: Unclassified

Summary:

It has been previously shown that hydrazine injected subcutaneously into rats, 2×10^{-3} moles per Kg., produces a periportal and midzonal fatty infiltration. In this study the ability of liver slices from hydrazine treated rats to convert amino acids into CO_2 and protein is presented. No significant loss of cellular protein could be detected. The capacity of liver slices prepared from rats treated with hydrazine to convert amino acids to CO_2 was depressed by approximately 60 to 70 per cent. No changes were found in either the general metabolic rate or conversion of acetate to CO_2 in liver slices from hydrazine treated rats. In contrast, the incorporation of labeled amino acids into cellular protein was increased.

These data suggest that hydrazine blocks the metabolic conversion of the amino acid to the corresponding keto acid, and the findings are consistent with the hypothesis that hydrazine blocks amino transfer reactions. Also, in contrast to experiments with carbon tetrachloride, hepatic steatosis in hydrazine treated rats does not appear to be a result of depressed protein synthesis.

BODY OF REPORT

Project No. 3A012501B813 Title Army Med. Basic Research in Life Sciences

Task No. 1 Title Biochemistry: Correlation of Morphologic and Chemical Changes in Cells Exposed to Toxic Chemicals

Description:

The general purpose of this study has been tp place on a biochemical basis the morphologic changes induced by toxic chemicals, as seen with the light and electron microscope. With the hypothesis developed in this work, the study of biochemical pathology can be extended to areas where no morphologic changes are seen, as is usually the case in studies on most toxic chemicals.

This particular study has focused on the effects of hydrazine, a toxic rocket propellant, upon metabolic function in the liver and kidney of rats

Progress: (Methods)

Osborne-Mendel rats weighing between 180 and 260 gm. were fed ad libitum on a normal diet of Purina laboratory chow. In each experiment, unless otherwise indicated, 12 rats were used in both experimental and control groups. Prior to starting the experiment, the rats were fasted for 18 hours. A 0.5 solution of hydrazine sulfate was made up daily and neutralized to pH 7.0 prior to injection. A dose of 2×10^{-3} moles per kg. was injected in the subcutaneous tissue of the right rear leg. Five hours later the animals were decapitated, and the livers excised and placed in ice-cold 0.9 per cent saline. Slices approximately 0.3 mm. thick were

prepared from the liver by the free hand technique. Tissue slices weighing approximately 150 mgm. from control animals were placed in control flasks; slices weighing approximately 180 mgm. were employed in the case of the hydrazine-treated animals. Preliminary experiments showed that the slightly larger weight of liver slices used from the hydrazine-treated animals compensated for the liver enlargement produced by hydrazine and yielded approximately the same amount of DNA in flasks from both experimental and control groups.

Standard Krebs Ringer bicarbonate buffer, pH 7.4, containing 1×10^{-3}M glucose was modified as follows: In the experiments with glycine, glycine was added directly to the buffer to give a final concentration of 1×10^{-3}M, and approximately 50 μc/L of glycine-1-C^{14} was added as tracer. In experiments with alanine, DL-alanine was added to the buffer to give a final concentration of 2×10^{-3}M; 50 μc/L of DL-alanine-1-C^{14} was added as tracer. In experiments with acetate, acetate was added to the buffer to give a final concentration of 1×10^{-3}M; 20 μc/L of acetate-1-C^{14} was added as tracer. The liver slices were placed in 25-ml. Erlenmeyer flasks with 3 ml. of cold buffer. The flasks were then sealed and flushed for 3 minutes with a mixture of 95 per cent O_2-5 per cent CO_2 through the inlet and outlet needle vents. The needles were then sealed with short lengths of knotted rubber tubing and the flasks placed in a Dubnoff metabolic shaking incubator with a shaking rate of 120 per minute for 1 hour at 37°C.

For $C^{14}O_2$ measurements, 1 ml. of a Hyamine solution was injected into the center well through the outlet needle at the end of the hour. One milliliter of 2 N sulfuric acid was then injected through the inlet needle into the buffer to release the CO_2. The needle vents were then resealed and replaced in the metabolic shaker for another hour. At the end of this interval the Hyamine was transferred from the suspended center well to a counting vial.

Fifteen milliliters of scintillation fluid, prepared by adding 5 gm. of PPO and 0.3 gm. of POPOP to a 1-qt. bottle of toluene, was added to each vial. Counts were determined in a Packard Tri-Carb scintillation Counter for 30 minutes. Internal standards were then added and counted in all vials. Quenching was found to be small and consistent, varying from 3 to 8 per cent.

For the determination of labeled protein, the tissue slices were removed from the medium at the end of a 2-hour incubation. Only trace amounts of labeled protein were isolated from the incubating media. Protein was isolated from the tissue slices, plated on a filter disc, and counted in a Nuclear Chicago gas-flow counter. In addition, this labeled protein was recounted in the scintillation counter. In this latter method, quenching was found to be significant, but scintillation counting did allow a simple, indirect determination of the efficiency of the gas-flow counter, on which a standard curve was then constructed. The total trichloroacetic acid soluble C^{14} within the tissue slice was checked by adding 0.1 ml. of the first TCA supernatant to 2 ml. of Hyamine and then counting in the scintillation counter. This is reported as cellular C^{14}.

DNA was determined by the method of Schneider. Oxygen uptake was measured in a standard Warburg apparatus in a Krebs-Ringer phosphate buffer, pH 7.4, at 37°C. Two 15-minute readings were averaged.

(Results)

The appearance of the rat livers 5 hours after the administration of the hydrazine solution confirmed the findings reported previously. Grossly the livers showed a slight increase in size and weight, appearing somewhat more pale and yellow than the corresponding control livers. Microscopically, small droplets of lipid could be detected with an oil red O stain in the hepatic cells of the periportal and midzonal regions. In addition, it was noted that the lipid droplets progressively increased in size and number from

the portal regions to midzonal regions. The lipid was maximal at the midzonal regions, forming a relatively sharp demarcation from the lipid-free pericentral zones.

Liver slices prepared from the hydrazine-treated animals demonstrated a marked decrease in the capacity to convert amino acids to CO_2. Although the oxidation of alanine to CO_2 was far greater than the oxidation rate of glycine, in both instances hydrazine effected a proportional 60 to 70 per cent decrease in CO_2 production from the added amino acid. In contrast, the specific activity of the protein of the liver slices appeared slightly increased with glycine and substantially increased with DL-alanine in the slices from the experimental animals. To rule out the possibility that this increase in specific activity of the protein might be secondary to a decrease in total intracellular protein in the slices from the hydrazine-treated animals, the total isolated protein per flask was compared to the total DNA per flask. Although 180 mg., wet weight, of liver slices from experimental animals was compared against 150 mg. of liver slices from the control animals, no difference was found between the amounts of protein in the flasks from the control and experimental animals. Since the weight of wet tissue initially placed in each flask was adjusted to give equal amounts of DNA in all flasks, the protein/DNA ratio was unchanged, indicating no significant loss of protein from the hepatic cell. An actual increase in the rate of incorporation of labeled amino acid into the protein of the slices from the experimental animals was observed.

Oxygen uptake was studied to determine to what extend, if any, the depression in amino acid oxidation might be due to a general metabolic depression caused by hydrazine. No difference was found in the rate of oxidation between liver slices from control and hydrazine-treated animals. As a

further check on this finding, liver slices were incubated for 1 hour in 1 x 10^{-3}M acetate tagged 1-C^{14}. The $C^{14}O_2$ formed was then determined to measure the conversion of acetate to CO_2. No significant difference was found in the ability of liver slices from control and treated animals to convert acetate to CO_2.

(Discussion)

In the production of CO_2 from a specific amino acid, two fundamental metabolic processes are involved: (1) the removal of the amino group from the amino acid and (2) the oxidation of the resulting keto acid. The data presented in this study, though demonstrating a marked depression in the overall oxidation of amino acids by liver slices, do not permit a precise localization of this block. The lack of any effect of hydrazine upon the rate of O_2 uptake in the liver slices demonstrates that this depression of amino acid oxidation is not due to a depression of general metabolic rate in the liver cell, indicating that the functional integrity of the cell is not impaired. This finding is consistent with the lack of cellular necrosis observed in previous studies. The lesion is further localized by the studies with labeled acetate: Lack of any significant change in rate of oxidation of acetate suggests that the oxidation of keto acids, via the tricarboxylic acid cycle, is not impaired. All this points to the hypothesis that the removal of the amino group is the inhibited reaction in amino acid oxidation in hydrazine-treated animals. This hypothesis is consistent with the work of Lewis and Izumi who measured the ability of injected glycine and lactic acid to elevate the blood glucose in hydrazine-treated hypoglycemic rabbits. Although hydrazine intoxication had no effect on the conversion of lactic acid to glucose, the conversion of injected glycine to blood glucose was impaired. Preliminary studies now in progress in this laboratory have shown depression of liver transaminase activity in hydrazine-treated rats.

A block at this level, i.e., transamination and deamination, also presents a possible mechanism for the hypoglycemia observed in some experimental animals, as blood glucose is dependent upon this conversion of amino acids to keto acids once the glycogen reserves are used.

Other workers using in vitro systems, have shown that hydrazine and its derivatives depresses the conversion of amino acids to keto acids. Since these reactions are reactivated by the addition of pyridoxal phosphate to the system, the reaction of hydrazine or a derivative with pyridoxal has been suggested as the blocking mechanism in these studies. This hypothesis could also account for the findings presented in this study, although further data are needed on this point.

The rate of incorporation of amino acids into cellular protein of the liver slice is increased in hydrazine-treated rats. It is likely, therefore, that the transport of the added amino acid into the intracellular pool is not impaired by hydrazine. The increased amounts of C^{14} from the labeled alanine found in the soluble TCA supernatant support this hypothesis. One mechanism for this increased incorporation of label into the cellular protein may be a mass-action effect secondary to an increase in the concentration of intracellular amino acid, the result of a block in the oxidative pathways in amino acid metabolism. An actual increase in the rate of intracellular transport of these amino acids, similar to the increased transport of p-aminohippuric acid effected by hydrazine in the kidney, is also possible.

Experiments with CCL_4-treated animals have consistently shown that one of the earliest lesions in the liver is a depression in protein synthesis. It has been suggested that this depression of protein synthesis in these experimental systems is also reflected in depressed lipoprotein synthesis, thereby reducing the outward transfer of lipid from the hepatic cell. Experiments with ethionine have also given support to this hypothesis. It is clear that in hydrazine intoxication, the mechanism of the hepatic steatosis

is not likely to be in the synthesis of the protein moiety of the lipoprotein. It should be emphasized that a block in some other step in the outward transfer of lipid is still a possibility. It is clear, though, that toxic injury to the liver, with resulting lipid accumulation, does not necessarily imply a depression in protein synthesis as the final common mechanism in hepatic steatosis. Significantly, both *in vivo* and *in vitro* preliminary studies in this laboratory have shown a marked increase in triglyceride synthesis from labeled fatty acids in the livers of hydrazine-treated rats.

(Conclusion Summarized)

Hydrazine injected subcutaneously into rats, 2×10^{-3} moles per kg., produces a periportal and midzonal fatty infiltration. No significant loss of cellular protein could be detected. The capacity of liver slices prepared from rats treated with hydrazine to convert amino acids to CO_2 was depressed by approximately 60 to 70 per cent. No changes were found in either the general metabolic rate or conversion of acetate to CO_2 in liver slices from hydrazine-treated rats. In contrast, the incorporation of labeled amino acids into cellular protein was increased. These data suggest that hydrazine blocks the metabolic conversion of the amino acid to the corresponding keto acid, and the findings are consistent with the hypothesis that hydrazine blocks aminotransfer reactions. Also, in contrast to experiments with carbon tetrachloride, hepatic steatosis in hydrazine-treated rats does not appear to be a result of depressed protein synthesis.

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ANNUAL PROGRESS REPORT

Title Page

Project No.: 3A012501A806 - Geographic Pathology

Task No. 2

Name & Address of Reporting Installation:

Armed Forces Institute of Pathology, Washington 25, D.C.

Name of Department and Division:

Geographic Pathology Division
Department of Pathology

Period Covered by the Report: 1 July 1962 - 30 June 1963.

Professional Authors of the Report:

Principal Investigator:	C. H. Binford, M. D. Chief, Geographic Pathology Division
Assistant:	D. J. Winslow, M. D. Chief, Infectious Diseases Branch

Reports Control Symbol: RCS-MEDDH-266

Security Classification: Unclassified

ABSTRACT

Project No.: 3A012501A806 Title: Communicable Disease

Task No. 2 Title: Geographic Pathology

Name & Address of Reporting Installation:

Armed Forces Institute of Pathology
Washington 25, D. C.

Period Covered by the Report: 1 July 1962 - 30 June 1963

Author:

C. H. Binford, M. D.

D. J. Winslow, M. D.

Reports Control Symbol: RCS-MEDDH-288

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SUMMARY

The Geographic Pathology Program of the AFIP enables the Staff of the Armed Forces Institute of Pathology to participate in research and teaching of infectious diseases of special importance to the military forces of the United States and its Allies.

A program in Kampala, East Africa, includes research on mycobacterial ulcer (Puruli ulcer), onchocerciasis, deep mycoses of East Africa, big spleen disease, endomyocardial fibrosis, and histopathologic documentation of the diseases of the area by detailed analysis of 200 autopsy cases.

A program in the Bangkok SEATO Laboratory is centered on research in opisthorciasis, determining the nature of pathologic lesions in wild animals, furnishing pathology support for other scientists, and studying the pathology of human diseases as seen at the Thai Army Hospital in Bangkok.

Staff members of the AFIP are conducting research projects in onchocerciasis and other filarioid diseases, Chagas' disease, leishmaniasis, viral hepatitis, and nutritional diseases.

BODY OF REPORT

Project No. 3A012501A806 Title: Communicable Diseases

Task No. Z Title: Geographic Pathology

DESCRIPTION

The primary mission of the Geographic Pathology Division of the AFIP has continued on the lines previously recorded, namely, to develop and operate a program that brings the staff of the AFIP into active participation in research and teaching of the infectious and other diseases which are of special importance to the military forces of the United States and its allies.

The organization of the Geographic Pathology Division of the AFIP has remained the same, comprising seven branches:

Geographic Pathology	Nutritional Pathology
Infectious Diseases	Leprosy
Bacteriology and Immunology	Geographic Zoonoses
Virology	

These seven Branches receive basic financial support from the AFIP and research support from several sources: (1) the Research and Development Command contracts; (2) a research grant from the National Institutes of Health (through the Leonard Wood Memorial); (3) a research grant from U.S. Veterans Administration; and (4) the U.S. Air Force.

The research program of the Geographic Pathology Division has been primarily oriented toward infectious diseases that are of military and international importance. Other activities of the Branches of the Geographic Pathology Division include research in nutritional diseases, teaching of the pathology of infectious diseases of geographic importance and diagnostic consultation on infections, especially those not commonly seen in the U.S.A.

I. RESEARCH ON DISEASES OF EAST AFRICA

The project "Research on Communicable Diseases and General Pathological Studies on Local Diseases," which has been supported by an R & D Grant to Makerere University College, Kampala, Uganda, has continued with Dr. Daniel H. Connor remaining as pathologist assigned from the Geographic Pathology Division of the AFIP.

In addition to other duties, he has furnished support to the parasitology program of WRAIR which has been established at Kampala.

Dr. Connor, in addition to carrying on research activities in Kampala, has undertaken several co-operative projects with members of the AFIP staff and has furnished the Staff material from many interesting cases illustrating diseases of East Africa.

A. RESEARCH ACTIVITIES IN UGANDA

1. Mycobacterial ulcer (Buruli ulcer)

Within the past few years work by Dr. H. F. Lunn of the Department of Surgery at Makerere University College Medical School and collaborators have identified the etiology of certain tropical ulcers occurring in Uganda as a Mycobacterium which is similar to the strains of Mycobacterium that have been reported as the cause of certain ulcers in the Congo. These African strains have many characteristics in common with Mycobacterium ulcerans that was first discovered in Australia.

Because these ulcers were first found in the Buruli district of Uganda, they have been called "Buruli" ulcers. More than 100 cases have now been reported. The ulcers occur on the limbs, trunk, neck, and face without obvious preference and in some patients cause osteolytic lesions. Undetermined are the natural reservoir and the mode of transmission.

Dr. Connor, in addition to collaborating with Dr. H. F. Lunn by making detailed histopathologic studies of the mycobacterial ulcers treated at the Mulago Hospital, is collaborating with Dr. Lunn, Captain N.E. Wilks, MSC, USA (Parasitologist) and Dr. M. H. King (microbiologist) in field studies designed to shed some light on the reservoir of infection and the mode of transmission. In February he participated in a field survey made in the Buruli ulcer territory (between the Victoria Nile and Lake Kyoga). In May he accompanied Dr. Lunn on a field study in the West Nile region where, at the Mogo Hospital, the staff had recognized approximately 100 cases. On this visit Dr. Lunn and Dr. Connor confirmed the presence of many cases of the disease in the West Nile area, photographed many lesions, and collected data on the environs of the homes of the patient. A plan was proposed for a study to determine, if possible, the natural reservoir of the Mycobacterium and mode of spread. Mycobacterial and histopathological studies of the lesions will be made.

Dr. Connor's collaboration with Dr. Lunn and others at Makerere has resulted in his sending to the AFIP histopathological material and clinical photographs on a number of cases of Buruli ulcer. This material has been used in the teaching of the pathology of tropical diseases.

2. Onchocerciasis

In collaboration with the parasitologist and entomologist from WRAIR Dr. Connor has made two extensive field trips to furnish pathology support for epidemiologic and ecologic studies of onchocerciasis. Skin snips, photographs of skin changes and skin nodules were made, medical histories taken, and physical examinations made. Among those examined were 42 dwarfes (Nakalanga) who were studied because of a possible relationship between "infestation with Onchocerca volvulus and the developmental abnormalities which cause these people to be classed as 'Nakalanga'."

In collaboration with Dr. Elson B. Helwig, Chief of the Skin and Gastrointestinal Pathology Branch of the AFIP, histopathologic studies are under way to determine if a correlation can be found between the wrinkling and sagging of the skin and histopathological and histochemical skin changes. This investigation is being carried out by the use of special histochemical stains such as aldehye fuschin, silver methods for reticulum, acid mucopolysaccharide with and without hyaluronidase, and periodic acid-Schiff with and without diastase.

3. Deep Mycotic Diseases of Uganda

Dr. Connor has collaborated with Dr. King, microbiologist, and other staff members at Makerere in the study of several cases of interesting mycotic diseases occurring in Uganda. Through Dr. Connor, the assistance of Dr. C. W. Emmons, Chief of the Mycology Section, NIH, and Special Consultant to the AFIP, has been obtained in identifying cultures of fungi from lesions studied by the Microbiology and Pathology Departments of Makerere. Of special significance has been the histopathological and mycologic study of a case of a disseminated infection caused by a fungus which morphologically and mycologically is similar to, if not identical with, Blastomyces dermatitidis, the cause of North American blastomycosis. Establishing the presence of this fungus in Africa would be of much interest to medical mycologists.

Another facet of this collaboration in mycology has been the submission to Dr. Emmons for identification agents isolated

from lesions of mycetoma. It is expected from this study that proper identification can be made of the Gram-positive filamentous fungi which are found in some of the mycetoma lesions of Uganda.

Dr. Connor has also submitted to the AFIP for further study clinical, photographic, and histopathologic data on several cases of subcutaneous phycomycosis due to Basidiobolus ranarum. Material from lesions due to Histoplasma duboisii has also been made available to the staff of the AFIP for study.

4. Big Spleen Disease

A disease of undetermined origin, characterized by splenomegaly and hepatic sinusoidal infiltrate, has been recognized for many years in the Mulago Hospital. In collaboration with the Department of Medicine, Surgery, and Hematology at Makerere Medical College a detailed investigation is being made of cases which are examined by biopsy or are studied at autopsy. Captain Wilks has been assisting in these studies by thoroughly investigating the possibility that in some cases unsuspected malaria may be associated with the splenomegaly and has identified P. malariae in two or three cases he has studied.

The investigations on "big spleen disease" cover complete hematologic studies including radioactive iron and chromium studies, liver biopsies, splenic biopsies, and lymph node biopsies. Detailed autopsy studies are done on fatal cases.

Since the initiation of this study the definition of this syndrome has been modified both clinically and pathologically, and it appears that more of the "big spleen disease" cases will be found to have more than one cause.

5. Analysis of Liver Biopsy Material

Because of the great variety of liver diseases occurring in patients of East Africa, an analysis is being made of the data obtained from routine liver biopsy examinations performed at Mulago Hospital.

6. Postmortem Pathology Study of Local Diseases

To establish data on the diseases causing death in Ugandan natives who die in Mulago Hospital, Kampala, Uganda, the results of 200 consecutive autopsies have been analyzed. Special attention was paid to the presence of gastro-intestinal parasites.

There had been no previous analysis in Uganda of autopsy data correlating cause of death, incidental findings with age, sex, and tribe. The study showed that of the 200 deaths, 23 were due to infectious diseases and 31 to neoplasm. Tuberculosis led the list of the infectious diseases causing death at this general hospital. Only 10 deaths were attributed to cardiac conditions. Endomyocardial fibrosis headed the list in this group (3 of 10).

The total contents of the large and small intestines were examined for parasites and ova. Exclusive of newborns the intestinal parasitic infection rate was 73.5%. The data obtained was tabulated for sex, age, place of residence, and tribe.

It is expected that the results of this detailed postmortem analysis of 200 cases will be prepared for publication.

7. Endomyocardial Fibrosis

This peculiar cardiopathy of Central Africa of unknown etiology is being studied thoroughly by the Medical and Pathology Staffs at Makerere Medical College. Dr. Connor is collaborating with Professor M.S.R. Hutt, Chairman, Department of Pathology, in these studies. Through the interest of Dr. W. C. Manion, Chief, Cardiac Pathology, AFIP, serum from eight of these cases has been studied by Colonel Elmer Chaffee, MSC, USA, Chief Immunology and Bacteriology Branch, AFIP, in order to determine if there are any serologic findings comparable to those found in chronic Chagas' disease. These serological studies have not shown evidence that Chagas' disease was present in any of these patients.

Detailed autopsy studies are being done on all cases of fatal endocardial disease. A thorough histopathologic investigation is made of labelled specimens from many anatomic sites of the heart. The histopathologic studies are being carried out by the use of many special mentods such as the phosphotungstic acid hematoxylin stain, Hart's elastica stain, Masson's trichrome stain, alcian blue stain, and Movat's pentachrome stain. In thoroughness of histopathologic investigation, this study should be outstanding and should serve to provide valuable information on the pathogenesis of this obscure endomycardial disease.

8. Nutritional Pathology Studies

In collaboration with Dr. Richard H. Follis, Jr., Chief of the Nutritional Pathology Branch of the AFIP, Dr.

Connor has been making detailed postmortem examinations on some of the cases of Kwashiorkor that die at Mulago Hospital, with special attention to renal changes and will make retrospective histopathologic examination of the kidneys on some of the cases of Kwashiorkor which have been autopsied at Mulago Hospital. Further study on this material will be done by Dr. Follis.

Dr. Connor has undertaken further collaboration with Dr. Follis by routinely weighing and studying histologically the thyroid gland obtained from autopsies at Mulago Hospital. To our knowledge no such definitive study has been made to determine the status of the thyroid gland in individuals dying in Africa.

9. Collaboration with Ophthalmic Branch, AFIP

Dr. Connor has undertaken in collaboration with Dr. Lorenz Zimmerman, Chief of the Ophthalmic Pathology Branch, AFIP, a selective study of eyes removed postmortem. At the end of May 1963, eyes from 28 cases had been sent to Dr. Zimmerman, who is carrying out the histopathologic examination. Dr. Connor supplies the clinical and postmortem data for each case. It is expected that a report will be made of the results of the examination of eyes from 100 selected cases autopsied at the Mulago Hospital. In addition, surgical eye specimens are sent through Dr. Connor. The purpose of these studies is to (1) correlate pathologic processes affecting the eyes with those affecting other tissues, (2) learn something of the frequency with which the eyes show varying degrees of damage as a result of other diseases not related to the patient's terminal illness (e.g., trachoma, onchocerciasis, trauma, etc.), and (3) compare eyes obtained postmortem in Africa with those obtained postmortem in this country.

Dr. Zimmerman cites one example of how this program is broadening our educational experience and how much experience may prove beneficial to patients in this country:

> ..."Among the surgical specimens we have received from African patients (specimens obtained by Dr. K.G. Hadija, Kampala, Uganda, and contributed through Dr. Connor by Professor M.S.R. Hutt of Makerere University College) is a group of peculiar granulomatous lesions involving the lids, conjunctiva, and orbit. Several of these have had fragments of a parasite believed by Major Donald L. Price to be a larval stage of Armillifer armillatus. Snakes of the Python and Bitis genera are definitive hosts. Ova are discharged from the snakes via the digestive

tract and bronchial secretions. Man gets infected by ingestion of ova. Tribes that have regular contact with snakes, e.g., eating of snake meat or use of living snakes as a fetish, have a high incidence of infection. Very recently we have seen in a conjunctival biopsy specimen sent in from Texas a granulomatous lesion with eosinophilia (but without parasites) that closely resembled the lesions received from Africa. A. armillatus probably is not known to exist in the U.S.A., but the closely related orthopod, Porocephalus crotali, is found in America. Therefore, we should anticipate seeing a similar lesion particularly in those areas where the soil might be contaminated by excreta of snakes".

B. FIELD TRIPS BY DR. CONNOR

Knowledge of the people, geographic environment, and medical institutions of the area essential in carrying out the AFIP mession were, to a large extent, obtained by selected field trips.

1. In September a trip to Tororo, Uganda, was made by Dr. Connor with Dr. Elvio H. Sadun, WRAIR, during which time the personnel and laboratories of the East African Trypanosomiasis Research Organization were visited and knowledge gained of the research program on trypanosomiasis being carried out in East Africa.

2. In July Dr. Connor made a visit to Mwanza, Tanganyika, in the company of Colonel Joe M. Blumberg, Deputy Director, AFIP, during which time the East African Research Institution and the Ross Institute were visited and much information gained concerning current research on schistosomiasis.

3. The Municipal Hospital at Jinja, Uganda, where many infectious diseases are treated, has been visited by Dr. Connor on several occasions and the hospitals at Kabale and Maska have each been visited once.

4. The Colony for leprosy patients at Lake Bunyonyi in Kigezi Province was visited by Dr. Connor in September.

5. In December Dr. Connor accompanied Captain Wilks and Mr. Barnley on a two-week safari into the Karamoja district for the primary purpose of determining the presence or absence of onchocerciasis and its vectors. No clinical or biopsy

evidence of onchocerciasis was found and no Simulium damnosum or Simulium neavei were found. Many interesting observations were made on the diseases of that area. Tropical ulcer was found to be a very serious problem. During this safari Captain Wilks surveyed approximately 200 natives for malarial parasites and Mr. Barnley in addition to surveys for Simulium collected insects from the native houses.

6. Previous mention (A-1) has been made of the field trips in February and in May into the country where mycobacterial ulcers are endemic.

II. ESTABLISHMENT OF A CO-OPERATIVE AFIP-SEATO PROGRAM IN GEOGRAPHIC PATHOLOGY

In carrying out the original plans for supporting the Medical Research Programs of the Armed Forces in Foreign Countries, Captain Sylvanus W. Nye, MC, USAF, from the Geographic Pathology Division, AFIP, was assigned in January 1963 to the SEATO Laboratory in Bangkok, Thailand, where he is working under the direction of Colonel James L. Hansen, MC, USA, Director of the U.S.A. Component of the SEATO Laboratory.

In March 1963, Dr. Robert E. Stowell, Scientific Director, AFIP, and Dr. C. H. Binford, Chief of the Geographic Pathology Division, made a visit to the SEATO Laboratory for the prupose of developing with Colonel Hansen and Captain Nye plans for a co-operative research program of the AFIP and SEATO Laboratory to be carried out through Captain Nye.

Dr. Nye has begun a project on the pathogenesis of Opisthorchis viverrini, which concerns a study of a natural infection in cats obtained from the Udorn area. He also has undertaken a pathological survey of vertebrate fauna trapped in Udorn and Chiengmai regions. He is furnishing pathology support to scientists assigned from WRAIR to the SEATO Laboratory. He also is undertaking the study of biopsy and autopsy material from patients in the Udorn area.

At the SEATO Laboratory, which occupies space in the building of the Army Institute of Pathology, Dr. Nye is co-operating with Thai Army pathologists in studies on human biopsy and autopsy material.

To develop the histopathological techniques in the SEATO Laboratory, Mr. Walter McAllister, Head TEchnician, Geographic Pathology Histopathology Laboratory, AFIP, in June 1963, was assigned on TDY to Bangkok for a period of five weeks.

III. ONCHOCERCIASIS

In addition to the work in onchocerciasis which Dr. Connor is carrying out in collaboration with Captain Wilks in Kampala, Commander F.G. Steen, MC, USN, Geographic Pathology Branch, AFIP, has begun studies in this disease. As a preliminary step he has consulted with scientists in Mexico who are carrying out research studies in this disease. The prospects for developing a collaborative program in Mexico did not appear good. There is need for much basic investigation in the pathogenesis of onchocerciasis. Although it has been estimated that 20,000,000 people have this disease, autopsy studies have been very few (20 as of 1957). Thorough postmortem investigations are needed to determine the presence of adult worms in locations other than in skin nodules. From increased knowledge of the distribution of the worms within the human host, there should be a better realization concerning the effectiveness of nodule removal; and hopefully this knowledge would be useful in attempts to place the infection in laboratory animals.

In February, 1963, Commander Steen visited Dr. Carlos Tejada, Chief, Division of Pathology, Instituto de Nutricion de Centro America, Guatemala City, to explore the possibility of developing a co-operative program. Dr. Steen, after his visit, has submitted a detailed protocol which provides in collaboration with Dr. Tejada for a study to be undertaken by making possible postmortem examinations on natives of Yepocapa, an endemic zone approximately 60 miles from Guatemala City. Exploration is now being made of possible ways for the support of such a study within Guatemala. Dr. Tejada has expressed a keen interest in this project and a willingness to collaborate. This project can be started when a satisfactory plan for support has been developed and approved.

IV. AMERICAN TRYPANOSOMIASIS AND LEISHMANIASIS

Dr. Donald J. Winslow, Chief, Infectious Diseases Branch, and Colonel Elmer F. Chaffee, MSC, USA, Chief, Immunology and Bacteriology Branch, Geographic Pathology Division, have continued investigation on American trypanosomiasis and leishmaniasis. Progress has been made in the following areas:

A. EXPERIMENTAL PATHOLOGY

Experimental pathology up to the present time has been chiefly applied to investigations on American trypanosomiasis. Various strains of *Trypanosoma cruzi* have been inoculated into mice and hamsters with an attempt to observe the differences in strain behavior in these laboratory animals. One strain, called G-4, was obtained from a *Triatoma infestans* which Dr. Winslow brought from Brazil. This strain had been isolated from a case of acute human Chagas' disease and had been used to infect a guinea pig. This strain proved to be very virulent for suckling mice, producing extensive parasitization of heart, smooth

muscles, striated muscles, and central nervous system. In some cases it was found to invade peripheral nerves. The feeding habits of three different vector bugs were observed, and it is planned that with the development of better facilities additional studies will be aimed at the evolutionary cycle of T. cruzi within the gastrointestinal tract of the triatome.

In March of 1963 a controlled inoculation experiment was carried out on white mice, using four strains of T. cruzi in approximately equal dosages. Histologic examination is being carried out on heart, lungs, spleen, esophagus, stomach, small intestine, colon, liver, pancreas, kidney, bladder, ovary, brain, spinal cord, abdominal wall, peritoneum, diaphragm, sacrospinalis muscle, and mesentery. It is hoped that a basic standard host response to an optimal dosage can be established for different strains so that the pathologic effects can be predicted in later experiments designed for investigations of environmental factors and other factors introduced to artificially modify the infection.

In addition to investigations on laboratory animals, plans are now being made to study the effects of T. cruzi on naturally infected reservoir animals, particularly the raccoon in the Patuxent, Maryland, area. Raccoons found to be naturally infected are to be autopsied, and histologic examination of their tissues will be made. The T. cruzi-like organisms isolated from naturally infected raccoons will also be inoculated into laboratory animals to determine their virulence for these animals and their histopathologic effects. Since triatomes are scarce in the Patuxent area, it is also planned that other possible vectors will be searched for. Much of this work will be done in collaboration with Colonel Elmer Chaffee, and Major Donald Price of the AFIP, and Dr. Carlton Herman of the Patuxent Wildlife Research Center, Laurel Maryland.

B. PATHOLOGY OF HUMAN CHAGAS' DISEASE

As a result of contacts made in Central and South America, fourteen new cases of human Chagas' disease have been investigated by histopathologic methods, which have revealed, in the acute cases, invasion of heart, striated muscle, and central nervous system by Trypanosoma cruzi. One rare subacute case of Chagas' disease in a child has shown early fibrosis in the interstiatial tissue of the myocardium, which was severely affected by a marked inflammatory exudate and contained numerous leishmaniform bodies of T. cruzi within heart muscle fibers. As regards the chronic manifestations of human chagas' disease, five hearts, one megacolon, and one megaesophagus have been studied. These

contributions of material from human cases of Chagas' disease have been made by Dr. Thales de Brito, Institute of Tropical Medicine, Sao Paulo, Brazil; Dr. Humberto Menezes, University of Sao Paulo, Ribeirao Preto, Brazil; Dr. Karl Brass, Central University, Valencia, Venezuela; and Dr. Juan Herrera, Santo Tomas Hospital, Panama City, Republic of Panama.

Following the receipt and study of these cases of human Chagas' disease, using gross material, tissue sections, photomicrographs, and clinical photos, exhibits were prepared, lectures were given, and teaching material provided for a Course on Tropical and Exotic Diseases. The contributions thus have become useful in furthering a research program on Chagas' disease and interesting other scientists in possible avenues of approach in this field.

C. PATHOLOGY OF HUMAN LEISHMANIASIS

As a result of similar contacts made in Central and South America, material from cases of human leishmaniasis has been received in the Infectious Diseases Branch. Kala-azar, cutaneous leishmaniasis, and post-kala-azar dermal leishmaniasis are represented by material received from the Far East and from Africa as well as from Central and South America. In comparison with older material, tissues from recent cases of kala-azar and dermal leishmaniasis show considerable similarity. However, there are certain chronic granulomatous lesions of the skin which have histologic alterations consistent with chronic leishmaniasis but in which no organisms can be found. Such lesions may result from treatment or spontaneous cure and are of interest to study in regard to one phase of the complete evolution of the disease. In addition, the location of the organisms within the reticulo-endothelial system and phagocytic cells throughout the body sets this disease apart from Chagas' disease, in which the etiologic agent seems always to be myoneurotropic. The differentiation of leishmania from leishmaniform bodies of T. cruzi is also being studied using a variety of special stains.

The material received from cases of human leishmaniasis as in Chagas' disease has been used in lectures, exhibits, and a Course on Tropical and Exotic Diseases. Principal contributors of the material were Dr. Jacinto Convit, Caracas, Venezuela; Dr. Carl Johnson, Gorgas Memorial Laboratory, Panama Canal Zone; Dr. Thales de Brito, Institute of Tropical Medicine, Sao Paulo, Brazil; and Dr. Colin G. Berry, Kaduna, Nigeria, Africa.

D. SEROLOGIC AND IMMUNOLOGIC STUDIES

Further serologic studies by Colonel Elmer F. Chaffee, MSC, USA, with cultures of Leishmania tropica obtained from Dr. P.H.C. Manson-Bahr have corroborated the previous report that L. tropica can be differentiated from L. donovani and L. braziliensis obtained on a trip (supported by the R&D Geographic Pathology Contract) to Venezuela and Brazil during May and June of 1962, are being investigated. Present studies show that L. donovani from South America is antigenically similar to a strain from Africa; strains of L. braziliensis obtained have been found to be variable in their antigens. Of particular interest was the finding that a strain obtained from Venezuela and one from northern Brazil appear antigenically to be L. tropica.

Studies to improve the specificity of T. cruzi antigens by chemical and physical means have not been successful to date, since chemical fractionation has resulted in a loss of sensitivity, and physical techniques used have failed to produce the desired specificity.

Serologic studies of sera obtained by Dr. William C. Manion, AFIP, from 8 cases of idiopathic heart disease from Africa have shown no evidence of antibodies when tested by complement fixation with an antigen prepared from T. cruzi.

Cultures from four strains of T. cruzi have been prepared by Col.Chaffee for the inoculation of mice used in comparative pathology studies by Dr. Donald J. Winslow.

Studies to improve the specificity of T. cruzi antigens without loss of sensitivity will be continued. Studies to evaluate the use of immunofluorescence as a diagnostic aid in tissue from cases of kala-azar and Chagas' Disease will be continued. Further studies will be made to corroborate the evidence that some strains of L. braziliensis found in South America are veritably L. tropica. Further strains for study are needed as well as supporting serological tests.

It is also hoped that further serum specimens from cases of Leishmaniasis and Chagas' Disease may be obtained from South America to further our serological studies.

E. CARDIOVASCULAR STUDIES

Dr. W.B. Manion Chief of the Cardiac Pathology Branch has continued gross and histopathologic studies of hearts from individuals dying of Chagas disease and has compared the findings

with myocardial and endocardial lesions in patients without Chagas' disease. He has also been greatly interested in comparing the cardiac changes in Chagas disease with those observed in certain cases from Africa. Although the complement fixing antibodies studies performed by Col. Chaffee on sera from African patients have shown no antibodies to T. cruzi antigens, Dr. Manion will continue his investigations.

Dr. Manion is preparing a set of slides of the various heart diseases of geographic importance. About 20-25 entities are considered and already 500 of the slides have been prepared. It is hoped that the set will be completed by December 1963.

V. RESEARCH PROGRAM IN PARASITOLOGY

Donald L. Price, Major, MSC, USA, Parasitologist, who was transferred from WRAIR to the AFIP 1 May 1963 has been assigned to the Geographic Pathology Division. As mentioned previously, he will collaborate with Dr. Winslow and Dr. Herman on the study of Trypanosoma found in raccoons at the Patuxent Wildlife Research Center. Study on infections should enable confirmation of the hypothesis that the organism in the raccoons is T. cruzi.

Filarioid Diseases:

A program on the Development of Dirofilaria uniformis in Anopheles quadrimaculatus, started at WRAIR, is continuing under the Geographic Pathology Branch. In the initial results of the study, larvae were found to begin penetration of the gut wall as early as 15 minutes after the mosquito fed on an infected rabbit. Larvae, after leaving the gut, penetrated the fat body where they proceeded to develop. Larvae remained in the fat body until near the end of the second stage, when they became active. Migration toward the head of the mosquito began on the seventh day after exposure. At fifteen days most of the larvae had reached the infective stage and had migrated to the head or proximal region of the thorax of the mosquito.

Major Price hopes to use the experience and information gained by the study on Dirofilaria uniformis as a model in developing a study designed to elucidate the detailed life cycles of other filaroid parasites, especially Onchocerca volvulus in Simulium.

VI. VIROLOGIC RESEARCH

A. INFECTIOUS HEPATITIS

The laboratory study of viral agents associated with human infectious hepatitis was continued by T. O. Berge, MSC, USA, Chief of the Virology Branch, AFIP, along several lines.

While the major financial support for this study has been obtained through an Army Research and Development contract under the Johns Hopkins University (Dr. Ivan Bennett), continued support has been given by the Geographic Pathology Division for professional staffing.

1. Study of Candidate Agents. Repeated efforts were made without success to duplicate reported results of the Research Laboratories, Parke, Davis and Company with agents, media and cell systems provided. It was concluded that the Parke, Davis test system would not provide a useful laboratory tool for purposes of the present study until cultural conditions could be better defined, and work with these materials was terminated.

The San Carlos-6 agent provided by Dr. Eldon Davis of CDC, Phoenix, was identified as a "prime" strain of Adenovirus type 16. Screening tests with the SC-6 virus and sera from cases of infectious hepatitis showed presence of neutralizing antibody in a high proportion of specimens tested. On this basis, the strain was accepted as a promising candidate agent for further testing.

Two agents were recently received from Dr. Karol Hok of Cutter Laboratories. One agent was recovered from a non-irradiated fibrinogen fraction from human plasma and the second from a filtered fecal pool collected in an institutional outbreak of hepatitis. Both agents have been passed successfully in this laboratory. Neither agent has yet been identified, but both differ markedly from the San Carlos type virus in cultural characteristics and cytopathic effect.

An uncharacterized agent isolated by Schneider and Muirhead has also been received but not yet examined. This strain was stated to have been recovered from liver of a fatal case of infectious hepatitis.

2. Chimpanzee Study. Intramuscular inoculation of young chimpanzees with plasma from a proved case of infectious hepatitis and with San Carlos-6 tissue culture virus, respectively, induced hepatomegaly, elevations of SGOT and SGPT titers, and minor but definite histologic changes in liver. Inoculation of SC-6 by corneal scarification in a third animal resulted in serum transaminase rises but no conjunctivitis or detectable liver changes. These studies are continuing and are being extended to other agents.

3. Virus Isolation Studies. Twelve acute-phase sera were arbitrarily selected for intensive isolation trials, including eleven from the Korean series of Conrad and one from a severe IH case at Andrews AFB. Urine specimens from most of these cases were also worked up. Cell systems employed included the Davis continuous human embryonic lung, Detroit-6y, Wistar WI-26 and WI-38, primary embryonic kidney and lung, and African green monkey kidney tube cultures. Diploid human fetal kidney and lung were employed less extensively.

Agents showing CPE characteristic of an adenovirus were recovered from serum of 3 of the 12 cases tested and from urine of a fourth case. The serum isolates appeared to be more closely related to the prototype Adenovirus 16 than to Adeno 16' (San Carlos) in tissue culture neutralization tests. Homologous antisera have not yet been prepared for reciprocal tests. The adenovirus-like agent recovered from urine was not neutralized by either Adeno 16 or Adeno 16' antiserum (32 units tested against less than 10 TCD_{50} of virus), and has not yet been identified. Re-isolations have not been accomplished for confirmation.

A second type of agent, tentatively termed "Agent 2", has been recovered from serum of 5 cases, including 4 of the same cases from which adenoviruses were recovered. All agents have been passed from 3 to 5 times and appear to be established in tissue culture. The CPE shown by "Agent 2" differs markedly from that shown by the adenoviruses, including cell rounding, shrinking and disintegration. Some differences in host cell susceptibility have also been noted. Several cultures show CPE suggestive of a mixture of the two types of agents; attempts are in progress to purify these strains by agent separation by several techniques.

Re-isolation has been accomplished at least twice in the case of 3 of these agents; others have not yet been attempted. "Agent 2" has not been identified, nor animal pathogenicity tested. Two agents of this type have been found to differ from the usual enteroviruses in that they are inactivated by treatment for 4 hours in the cold with 50 per cent ether (7 log reduction in titer) and by 1:400 dilution of sodium desoxycholate.

Convalescent sera from cases from which these agents were recovered have not yet been tested for presence of homologous antibody. These tests will be carried out in the near future after virus mixtures have been separated. Relationship of these agents to infectious hepatitis has not yet been established.

4. Future Plans. Further viral isolation studies are planned after the present agent recoveries have been authenticated by re-isolation. Agent characterization will be pursued and precise identification made if possible. Attempts will be continued to obtain further candidate agents for study. Serologic tests will be expanded as suitable antigens can be prepared.

Further trials will be conducted in chimpanzees or monkeys (Erythrocebus patas) as feasible, employing the most promising candidate agents in attempts to reproduce the disease experimentally.

B. LABORATORY FOR ISOLATION OF VIRAL AGENTS IN GEOGRAPHIC MEDICINE.

It has been hoped that a special viral laboratory could be established which would permit the investigation of viral or possible viral agents sent in by field representatives of the Geographic Pathology Division. Because of lack of space at the AFIP, exploration has been made of the possibility of establishing such a laboratory in collaboration with the National Naval Medical Institute where space was available. No specific action has been taken on the establishment of this laboratory. Now it appears feasible, through the proposed reorganization of laboratory space at the AFIP, to begin a small virus isolation laboratory at the AFIP. It is hoped that if funds are available this can be activated within a few months.

VII. NUTRITIONAL PATHOLOGY

The program of R. H. Follis Jr., Chief of the Nutrional Pathology Branch has been to study in suitable experimental animals the anatomical and biochemical effects of deficient states. These investigations have been supported financially by other sources. During the past few years a good deal of attention has been directed to experimental goiter, protein-like syndrome, anemia and certain bone diseases. It has seemed highly desirable to gain first-hand knowledge of naturally occurring disease in man in various parts of the world. Dr. Follis has been a member of ICNND surveys in Vietnam (1959) and Thailand (1960). Last year (1962) through support from this contract he had the opportunity to obtain first hand experience concerning those nutritional diseases which are so prevalent in Africa. In the spring of 1963 visits were made to various laboratories and hospitals in Central and South America. Here endemic goiter, protein malnutrition, anemia, and the interrelations of infection and nutrition were observed and discussed with workers active in these fields.

VIII. DOCUMENTATION OF DISEASES IN SELECTED GEOGRAPHIC AREAS

During the year material from 1217 Geographic pathology cases were acquired by the AFIP from contributors outside of the USA (exclusive of U.S.Military installations).

TOTAL GEOGRAPHIC PATHOLOGY CASES 1217

By /reas:

Asia	270	Europe	47
/frica	294	Iceland	61
/ustralia	6	Philippines	124
Canada	79	South /merica	2.1
Caribbean Sea	42	Turkey	4
Central /merica & Mexico	39		

/mong the special contributors of pathologic material from various geographic areas have been:

Prof. M.S.R.Hutt and Dr. D. H. Connor	Uganda
LCDR James Fresh,MC,USN	Taiwan
Dr. Clarence /elat	Ghana
Dr.Colin G.Berry	Nigeria
Prof. J.P.Becker	S. /frica
Dr. J. P. Wiersema	Surinam
Dr. /inod G.Daftary	India
Dr.Thales de Brito and Dr.Humberto Menezes	Brazil
Prof.J.Convit and Prof. Karl Brass	/enezuela
Prof.Niels Dungal	Iceland
Leonard Wood Memorial	Philippines

IX. POSTGR/DU/TE COURSE ON THE P/THOLOGY OF TROPIC/L /ND EXOTIC DISE/SES.

/ five-day Course on the Pathology of Tropical and Exotic diseases, directed by Dr. D. J. Winslow, was presented to approximately 35 registrants on /pril 1-5, 1963, at the /FIP /nnex. The Course included in its subject material protozoal, helminthic, bacterial, viral, and mycotic infections. Emphasis was given to microscopic study by including boxes of slides and a microscope for each registrant. The faculty of 20 was obtained from the /FIP Staff, WR/IR and R&D Staff, and medical schools. This course was very well received by the registrants, most of whom were pathologists.

X. TE/CHING /IDS IN GEOGR/PHIC P/THOLOGY.

During the year 35 100-microslide study sets on tropical and exotic diseases were prepared by Dr. Winslow and Dr. Stean. The syllabus for this set is in press and will be published shortly. These sets are being made available to the registrants of the Course on the Pathology of Tropical and Exotic Diseases and to special contributors who have supplied blocks and fixed tissue as well as

clinical abstracts. The sets will be made available to the American Registry of Pathology for loan to interested pathologists.

XI. HISTOPATHOLOGY RESEARCH LABORATORY FOR GEOGRAPHIC PATHOLOGY.

The special Histopathology Research Laboratory for Geographic Pathology has prepared the human and experimental histopathologic preparations used in the Geographic Pathology Program. In addition to supporting the Geographic Pathology Division in Washington, considerable assistance has been given to Dr. Connor on research studies being carried out in Africa.

During the past year 26,941 slides were prepared and stained -- among these 11,458 were specially stained preparations.

SCIENTIFIC EXHIBITS

Exhibits in geographic pathology, with part support from the R&D contract, were displayed at the following meetings:

1. The IV Congress, International Academy of Pathology, Zurich, Switzerland, 8-13 July 1962:

   "Transmission of Mycobacterium leprae to Animals - Nerve Involvement in the Golden Hamster (Cricetus Auratus)."
   Chapman H. Binford, M. D.
   Russell M. Madison, Lt. Col., USAF, MC

   "Mycetoma"
   Donald J. Winslow, M.D.
   Frank G. Steen, Cdr., MC, USN

   "Pathology of African Horsesickness"
   Fred D. Maurer, Col., USA, MC
   R.M. McCully, Captain, USAF, MC

2. The American Society of Tropical Medicine and Hygiene Eleventh Annual Meeting, Atlanta, Georgia, 31 October - 3 November 1962:

   "Transmission of Mycobacterium leprae to Animals - Nerve Involvement in the Golden Hamster (Cricetus Auratus)."
   Chapman H. Binford, M. D.
   Russell M. Madison, Lt. Col., USAF,MC

"Mycetoma"
Donald J. Winslow, M. D.
Frank G. Steen, Cdr., MC, USN

"Metazoan Parasitic Diseases in Man and Animals"
M. A. Ross, Lt. Col., USA, VC
R. M. McCully, Captain, USAF, VC

3. The International Academy of Pathology and the American Association of Pathologists and Bacteriologists, Cincinnati, Ohio, 26 April - 1 May 1963:

"Geographic Infectious Diseases"
Donald J. Winslow, M. D.
Frank G. Steen, Cdr., MC, USN

4. The American Medical Association Meeting, Atlantic City, New Jersey, 17-21 June 1963:

"Geographic Infectious Diseases"
Donald J. Winslow, M. D.
Frank G. Steen, Cdr., MC, USN

SELECTED PUBLICATIONS BY STAFF MEMBERS AFIP IN GEOGRAPHIC PATHOLOGY AND INFECTIOUS DISEASES:

Binford, C. H.: Tissue reactions elicited by fungi, in Fungi and Fungous Diseases, Symposium of the Section of Microbiology, The New York Academy of Medicine, edited by Dalldorf, G., Springfield, Ill., Charles C.Thomas, 1962 chap. 16, pp. 220-238.

Binford, C. H.: Studies on a Mycobacterium obtained from the golden hamster (Cricetus auratus) after inoculation with lepromatous tissue, 942-955,Laboratory Investigation,Vol.11,1962.

Straatsma, B. R., Zimmerman, L.E., and Gass, J.D.M.: Phycomycosis: a clinicopathologic study of fifty-one cases, 963-985, Laboratory Investigation, Vol. 11, 1962.

Barile, M. F., Blumberg, J. M., Kraul, C.W. and Yaguchi, R.: Penile lesions among U.S.Armed Forces personnel in Japan, the prevalence of herpes simplex and the role of pleuropneumonia-like organisms, Arch. Derm. (Chic.) 86: 273-281 (Sept.) 1962.

Cross, R. M. and Binford, C. H.: Is Nocardia asteroides an opportunist? Lab. Invest. 11: 1103-1109 (Nov.,pt.2) 1962.

Fine, B. S.: Intraocular mycotic infections, Lab.Invest. 11:1161-1171 (Nov., pt. 2) 1962.

Follis, R. H., Jr.: The ecologic approach to nutritional disease, Nutr. Rev. 20: 193-195 (July) 1962.

Zimmerman, L. E.: Mycotic keratitis, Lab. Invest. 11: 1151-1160 (Nov., pt. 2) 1962.

Follis, R.H.,Jr.: The Ecology of Hunger, Milit. Med. 128: 384-391 (May) 1963.

Maurer, F. D.: Emerging Animal Diseases, Milit. Med. 128: 327-333 (Apr.) 1963.

Maurer, F.D.,and McCully,R.M.: African Horse-Sickness-With Emphasis on Pathology,Amer. J.Vet. Res. 24: 235-266 (Mar.) 1963.

Smetana,H.F.: Yellow Fever,60 Years Later, Milit.Med. 128: 306-316 (Apr.) 1963.

Zimmerman, L. E.: Keratomycosis, Survey Ophthal. 8: 1-25 (Feb.) 1963.

A successful course in the pathology of exotic and tropical diseases was given to pathologists, using a microscopic slide teaching set especially prepared for the course. Members of the AFIP staff have prepared several exhibits in geographic pathology and have published a number of papers in the field of geographic pathology and on infectious diseases of importance to military medicine.

ABSTRACT

PROJECT NO. 3A012501B813 TITLE: Physiology

TASK NO. 3 TITLE: Ultrastructural Changes in Muscle Injury

NAME AND ADDRESS OF REPORTING INSTALLATION:

Armed Forces Institute of Pathology
Washington 25, D. C.

PERIOD COVERED BY THE REPORT: 1 July 1962 - 30 June 1963

AUTHORS: Joe M. Blumberg, Colonel, MC, USA
Harold M. Price, Captain, MC, USA
Edward L. Howes, Jr., Captain, MC, USA

REPORTS CONTROL SYMBOL: RCS-MEDDH-288

SECURITY CLASSIFICATION: Unclassified

SUMMARY

With the rapid advancement of electron microscopic techniques in the last 10 years, there has been a parallel increment in our knowledge of the normal submicroscopic structure of the muscle cell. A complex array of macromolecular structures has been demonstrated which was concealed by the relatively low resolution of the conventional microscope. These electron microscopic studies of the normal imply a need for a detailed re-examination of the basic morphologic responses of muscle to injury on a submicroscopic level. It has become evident that the pathologic alterations within the injured muscle cell must be more clearly defined in terms of the submicroscopic structures of the

cell and correlated with known physiologic and biochemical data. To date only a few ultrastructural studies of the injured muscle cell have been reported. Their results are limited, but they raise a number of questions that can only be answered by more thorough studies of each phase of muscle injury. The purpose of this project has been and will be in the next few years to study the ultrastructure of injured and diseased skeletal muscle in man and animals.

In our work to date we have gained some insight into the general changes manifested by cellular organelles within injured and regenerating muscle cells. The basement membrane of the muscle fiber was observed to be the most resistant component of the cell to cold injury. It formed a micro-skeleton (or sarcolemmal tube) in which regeneration of the injured muscle fiber took place. The plasma membrane as well as myofibrillar differentiation were lost within 24 hours after injury. The mitochondria and sarcoplasmic reticulum underwent a variety of nonspecific degenerative changes. Regeneration was first manifested after 72 hours by the appearance of a mononuclear undifferentiated cell (or "early myoblast") whose cytoplasm contained numerous, randomly arranged, uniform, fine filaments. These filaments were intimately associated with ribonucleoprotein particles. With time, binuclear and eventually multinuclear forms of these cells were seen; and within their cytoplasm progressive myofibrillar differentiation was apparent. The large "actin" and "myosin" myofilaments making up the myofibrils were thought to be formed by polymerization of the fine filaments found within the cytoplasm of the "early myoblasts."

With the eventual development of a mature muscle fiber the "old" basement membrane which formed the sarcolemmal tube appeared to disintegrate and a "new" basement membrane was formed in juxtaposition to the newly regenerated fiber.

A study of the ultrastructure of the developing muscle fiber in the embryonic state has recently been initiated. This study will aid us in gaining insight into the regenerative response of skeletal muscle to injury.

Examination of the fine structure of the skeletal muscle of albino rats on a chronic vitamen E deficient diet has demonstrated a probable intimate important relationship between the formation of ceroid pigment and the sarcoplasmic reticulum. In addition, injured portions of sarcoplasm appeared to isolate themselves within the cell by single membrane-limited structures from other less affected areas of the cell.

A technique for obtaining well fixed human skeletal muscle biopsy specimens for electron microscopic examination has been developed. This necessitated the development of a special stainless steel clamp. Material has been obtained from 17 patients, both at Walter Reed Army Medical Center and UCLA Medical Center, Los Angeles, California. Preliminary observations indicate the specimens are well fixed for submicroscopic studies.

The proposed objectives of this project are:

1. To examine the ultrastructure of the degenerative and regenerative changes in injured skeletal muscle.

2. To study the embryological development of skeletal muscle on an ultrastructural level.

3. To define the submicroscopic changes in experimentally-induced skeletal muscle alterations of military significance (i.e., nutritional deficiencies, thermal lesions, infectious agents, radiation injury, fatigue).

4. To develop techniques for the processing of human muscle biopsy material so as to get the best possible fixation for electron microscopic examination.

5. To study the normal ultrastructure of human skeletal muscle.

6. To examine the submicroscopic alterations induced by injuries and natural diseases that affect skeletal muscle in man.

PROGRESS

A. Background information

Technical difficulties and a change of personnel made it impractical for us to proceed with the plans as outlined in our previous application for research funds for Fiscal Year 1963. The program was mainly based on the possibility of attaching ferritin to specific neurotoxins and pharmacological agents which affect the motor end-plate. Progress along this line was slow and inconclusive. In view of these difficulties we have altered the major emphasis of our research endeavors

from the neuromuscular junction to the muscle fiber itself, with our specific aims being those outlined above. The interdependence of the neuromuscular junction and the muscle fiber, however, make it a necessity to understand the basic pathologic changes that both are subject to; therefore, as progress permits in our investigation and techniques are developed, we will again pursue problems pertaining to the motor end-plate.

B. Observations of degenerative and regenerative changes in skeletal muscle following injury by cold.

1. An injury by cold results in an excellent biological model with which to study both the acute degenerative as well as regenerative changes in skeletal muscle. The anterior tibial muscles of adult Long Evans rats were injured by the superficial application of a brass rod dipped in liquid nitrogen to the muscle surface for 10 seconds. The animals were sacrificed at time periods varying from 15 minutes to 21 days after injury, and the tissue was studied by electron microscopic, phase, and regular light microscopic techniques.

2. By the end of 24 hours the typical sarcolemmal tubes known to the muscle pathologist were evident under routine light microscopic observations. By electron microscopic examination it was evident that these sarcolemmal tubes were formed by the basement membranes of the injured muscle cell; the true cell membranes apparently had undergone degeneration. There was a complete lack of myofibrillar differentiation and cross striations. The remaining myofilaments were fragmented.

The mitochondria manifested a variety of nonspecific degenerative changes which other investigators have described in tissues such as liver, kidney, and brain. There were numerous abnormal membranous-like structures that appeared to be related to the degenerating endoplasmic reticulum, or may have possibly been derived from phospholipids freed from other degenerating cytoplasmic components.

3. Within the degenerating and early regenerating periods, prominent numbers of macrophages were seen within the sarcolemmal tubes. On a submicroscopic level the cytoplasm of these cells was found to contain a variety of structures: rough-surfaced endoplasmic reticulum, numerous cytosomes, lipid particles, Golgi structures, and fine filaments. The fine filaments averaged 70 to 80 Å in diameter, were arranged in bundles and did not appear to be associated with ribonucleoprotein particles. The nuclei of these macrophages lacked prominent nucleoli and were lobulated and irregular in shape.

4. Within the sarcolemmal tubes, 72 hours after injury, were found a significant number of fusiform, mononuclear cells that were located peripherally alongside the "old" basement membranes that formed the tubes. The cytoplasm of these cells contained an abundant number of very fine, randomly scattered, homogeneous filaments that averaged 50 to 60 Å in diameter and were very intimately associated with ribonucleoprotein particles. Experimental evidence published to date by several molecular biologists indicates that ribonucleoprotein particles are the possible sites of protein formation within a cell. If this

is true, these fine filaments may be the actin, myosin, and tropomyosin building blocks that make up the myofilaments of the mature muscle fiber. Also within the cytoplasm were seen mitochondria, a prominent number of Golgi structures, and an occasional rough-surfaced endoplasmic reticulum form. The nuclei of these cells contained several prominent nucleoli. We have tentatively designated these mononuclear cells as undifferentiated cell types, but believe them to be very early myoblasts. Their origin is uncertain.

5. With time, binuclear and eventually multinuclear forms of these cells were evident, and they had the general appearance under the light microscope of what some embryologists call "myotubes." Within these cells, under the electron microscope, rudimentary myofibrillar forms could be identified. These rudimentary myofibrillar forms were located peripherally in the cell. In the proximal nuclear area could still be seen many of the disorderly arranged fine filaments noted above in the undifferentiated cell type. It was thought that the actin and myosin myofilaments making up the myofibrils were formed by polymerization of these fine filaments which are apparently protein moieties in view of their close relationship to ribonucleoprotein particles.

6. By the 12th to 14th day myofibrillar differentiation was more advanced. I-bands with early Z-line formation were evident.

7. By the 18th to 21st days relatively mature muscle fibers were identified; at this time, however, two basement membranes were

often seen alongside these cells. The inner basement membrane appeared normal and was located in normal juxtaposition to the cell membrane of the newly regenerated muscle fiber. The outer basement membrane was fragmented and thrown into several folds. It probably represented the degenerating remnants of the basement membrane that had formed the micro-skeleton (or sarcolemmal tube) in which the newly regenerated fiber had formed.

8. In addition to the formation of the contractile structures within the developing muscle cells as described above, there were also several other prominent morphologic features evident. They all appeared to have very prominent Golgi structures. Nuclear pores were more evident than usually seen within the mature muscle cell, and lipid bodies were also more prominent.

9. This study has given us a guideline by which to familiarize ourselves with the common acute degenerative changes of the cell organelles within the injured muscle fiber. We have also been able, for the first time to our knowledge, to define the ultrastructural morphology of the "discontinuous type" of skeletal muscle regeneration as represented by the development of the undifferentiated cell type. Using the same biologic model we plan to study the so-called "continuous or budding form" of skeletal muscle regeneration and thereby hope to gain some insight into the possible origin of the undifferentiated cell type. This experimental system also affords a good setting in which

histochemical and autoradiographic techniques may possibly be applied so as to more specifically define the formation of the intracellular protein structures seen within these undifferentiated cells that are presumed to be the precursors of the myofilaments seen within the better differentiated cell types.

C. Embryological development

An evaluation of the embryological development of skeletal muscle on an ultrastructural level was felt to be very necessary in order to fully understand the regenerative responses of injured skeletal muscle. No submicroscopic study of this type has been reported to date. The brook trout was chosen for our initial embryological studies because it has been fairly well studied by conventional microscopic methods; it is a relatively simple developmental form amenable to good electron fixation; and under artificial conditions in the fish hatchery it has a short gestation period (approximately a little over 30 days). Material was obtained from the Maryland State Fish Hatcheries and fixed from day one through day thirty-two by the conventional methods for electron microscopic study. The material has not been studied as yet; preliminary sampling, however, indicates that it is well fixed for electron microscopic examination. It is planned in the future, after some understanding of the development of the muscle fiber in the brook trout is gained, to study the embryological formation of skeletal muscle within a mammalian species.

D. Chronic vitamin E deficiency in the albino rat

1. Various states of malnutrition show evidence of pigment accumulation not unlike that of chronic vitamin E deficiency (alpha-tocopherol deficiency) in animals. The degenerative effects observed in skeletal muscle of such animals has been described by conventional microscopic studies. The combined value of the ultrastructure study of a specific metabolic alteration within the skeletal muscle and the invaluable comparison that could be drawn from our other studies with this chronic degenerative lesion made such a study worthwhile. Albino rats on a vitamin E deficient diet from 6 to 11 months were obtained from Doctor Klaus Schwartz of the National Institutes of Health. The soleus muscle of these rats was fixed for electron microscopy and conventional microscopy.

2. An early change consisted of a peripheral sarcolemmal nuclear proliferation and some central migration of nuclei. This was followed by the accumulation of pigment material about the nuclei. At time, within the injured muscle fiber, damaged parts of the cell appeared to be isolated from uninjured portions by the formation of large segments of membrane-limited cytoplasm that often contained one or two nuclei. At a later stage pigment-laden mononuclear cells were seen surrounded by fibrous tissue.

3. The pigment itself, not only within skeletal muscle, but also within smooth muscle of the uterus, assumed several forms. Single membrane-limited structures containing moderately electron-dense

homogeneous material, other material of varying electron density, or thick and thin "myelin-like" figures all were seen. Larger accumulations of pigment seemed to rise from coalescence of smaller membrane-limited structures. A recurring structure in proximity to this pigment was a vacuole defined by a markedly thickened single membrane, usually containing finely stippled material, and occasionally recognizable portions of mitochondria. Origin of this structure appeared to be from endoplasmic reticulum at the A-I band junction.

4. Occasionally nuclear changes were noted in the muscle cell. These changes consisted of multiple involutions and apparent nuclear inclusions of pigment material.

5. Breakdown of the myofibrils appeared to occur first at the site of the Z-line. Dissolution of the Z-line resulted with the appearance of thick strings of electron-dense material that seemed to lie parallel to the myofibril itself.

6. Many myelinated axons have been seen in the affected muscle and have not shown changes. One motor end-plate has appeared degenerated and a muscle spindle has shown pigment accumulation. Advanced muscle changes show replacement to a large extent by fibrous tissue within which are scattered pigment-laden macrophages and muscle cells with apparent loss of mass.

7. Contemplated future studies involve electron microscopic histochemical determinations for acid phosphatase activity to investigate the presence of lysosome-like structures and also a PAS stain

for electron microscopy to demonstrate what portions of the pigment are able to take up this stain.

E. Human muscle

It has become evident from working with animal material that optimal fixation of skeletal muscle for electron microscopic examination is achieved by fixing the muscle in-situ while the muscle is put under a moderate degree of stretch by manipulation of the animal's limb. This prevents marked contraction of the muscle fibers when they come in contact with the fixative. This technique is obviously impossible to use in gathering human material; therefore, it became necessary to develop a simple method for preventing marked contraction of the muscle fiber on contact with the fixative and at the same time for getting the specimen into fixative as rapidly as possible. A clamp was designed which can be manipulated by a single operator and which will hold a small muscle fragment under a moderate degree of tension when it is placed in the fixative. This clamp was made using a type of stainless steel that will not react chemically with the fixative. It was developed in the machine shop of the Armed Forces Institute of Pathology with the technical cooperation of SFC O. D. Hutson, USA.

With the help of Captain Robert Fitzgerald, USAF, MC, a neurological resident, and Major Darrell Buchanan, a neurologist, biopsies have been obtained from seven patients at Walter Reed General Hospital who are suffering from primary myopathies. In addition, with the

assistance of Dr. Carl Pearson, Associate Professor of Medicine at UCLA Medical School, and Dr. Sheldon Leonard, Associate Professor of Surgery at UCLA Medical School, material has been obtained from ten other patients seen at the UCLA Medical Center at Los Angeles, California. Six of these later patients have primary myopathies, three of them are siblings of patients with muscular dystrophy, and one is a "normal." Preliminary examination of this material to date indicates that it is adequately fixed for electron microscopic examination. We are just beginning to study these specimens in more detail.

Papers presented at scientific meetings:

Electron microscopic observations of muscle injury. Harold M. Price, Edward L. Howes, Jr., and Joe M. Blumberg. American Association of Neuropathologists (39th Annual Meeting) Atlantic City, New Jersey, 8 June 1963.

Publications in preparation:

Electron microscopic observations of muscle injury: I. Degenerative changes. Harold M. Price, Edward L. Howes, Jr., and Joe M. Blumberg.

Electron microscopic observations of muscle injury: II. "Discontinuous" regeneration. Harold M. Price, Edward L. Howes, Jr., and Joe M. Blumberg.

Ultrastructural observations on skeletal muscle in chronic vitamin E deficiency. Edward L. Howes, Jr., Harold M. Price, and Joe M. Blumberg.

New technique for fixing human skeletal muscle for electron microscopy. Harold M. Price, Edward L. Howes, Jr., Robert F. Fitzgerald, and Joe M. Blumberg.

Ultrastructural anatomy of skeletal muscle (Symposium on Muscle Disease). Harold M. Price. Am. J. Med. (late 1963).

ANNUAL PROGRESS REPORT

Title Page

Project No.: 3A012501A806 - Military Preventive Medicine

Task No.:4 Rapid Identification
Subtask: X-Ray Spectrochemical Analysis of Human Brain

Name & Address of Reporting Installation:

Armed Forces Institute of Pathology, Washington 25, D. C.

Name of Department and Division:

Pathology Department, Division B, Neuropathology Branch
Pathology Department, Division D, Histochemistry Branch

Period Covered by the Report: 1 July 1962 - 30 June 1963

Professional Authors of the Report:

Principal Investigator: Kenneth M. Earle, M. D., Chief of Neuropathology Branch, AFIP

Assistant: Frank B. Johnson, M. D., Chief of Basic Sciences Division and Histochemistry Branch, AFIP

Reports Control Symbol: RCS-MEDDH-288

Security Classification: Unclassified

ABSTRACT

Project No. 3A012501A806 Title Military Preventive Medicine

Task No. 4 Title Rapid Identification

Subtask Title X-Ray Spectrochemical Analysis of Human Brain

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology, Washington 25, D. C.

Period Covered by the Report: 1 July 1962 - 30 June 1963

Authors:

Kenneth M. Earle, M. D., Chief of Neuropathology Branch, AFIP

Frank B. Johnson, M. D., Chief of Basic Sciences Division and Histochemistry Branch, AFIP

Reports Control Symbol: RCS-MEDDH-288

Security Classification: Unclassified

Summary:

The initial period of this research project was spent largely in obtaining the General Electric XRD6 X-Ray Emission and Diffraction equipment, in installation, and in checking the component parts of the equipment. In addition, experiments were conducted in sample preparation and in powder diffraction patterns of crystals from human pineal glands using the Siemens Crystalloflex II equipment.

The X-Ray diffraction powder pattern of the crystals from twenty formalin fixed human pineal glands extracted with 1 N sodium hydroxide, revealed the pattern of an hydroxy apatite. We were concerned, however, by recent indications that the crystallinity may have been altered by the extraction with sodium hydroxide. Therefore, we plan to repeat the diffraction experiments using other methods of extraction of the crystals including blunt dissection. The diffractometer will be used to check the film diffraction pattern.

The long time required to obtain and to install the XRD6 unit has prevented us from conducting any definitive experiments in X-Ray fluorescent spectrography during the period of this report.

BODY OF REPORT

Project No. 3A012501A806 Title Military Preventive Medicine

Task No. 4 Title Rapid Identification

Subtask Title X-Ray Spectrochemical Analysis of Human Brain

Description:

In view of the exposure of military personnel in all parts of the world to a wide variety of diseases and potentially toxic agents, new methods are needed for the rapid identification of potentially toxic elements and of pathological crystalline precipitates which may be found in diseased brains. Most of the techniques of wet chemical analysis of the brain require destruction of the tissue and lengthy procedures. X-Ray diffraction and X-Ray fluorescence emission spectroscopy provide non-destructive means for identification of crystals and for the qualitative and semi-quantitative analysis of all elements from Magnesium (Atomic number 12) through Uranium (Atomic number 92). Trace amounts of many of these elements can be detected if significant amounts of contamination of the specimen are carefully avoided. The specific aim of this project is to develop and to apply X-Ray fluorescence spectroscopy and X-Ray diffraction as methods for routine use in rapid identification of elements and crystals in the brain which may be the cause of crippling or fatal diseases of the nervous system. The development of new fuels, new weapons and new drugs requires that we develop new and rapid techniques for identification of the harmful effects of these agents upon the nervous system.

The first phase of this project will be to analyze crystals from human pineal glands as a means of developing the techniques and as a means of exploring the composition of pathological crystals in brain tissue. Crystals occur in the pineal gland in a majority of adults and these crystals can be extracted easily from the parenchyma of the pineal gland. Very little is known about the composition of these crystals or why they occur. After the basic techniques are established we shall extend these studies toward the development of techniques for analysis of bulk brain specimens and for analysis of specific areas of the brain in cases with neurological diseases of well defined and of unknown etiology.

Progress:

The request for this contract was submitted to the Surgeon General by the Director of the Armed Forces Institute of Pathology on 16 April 1962. Notice of award of funds was received on 21 September 1962 (effective 1 July 1962). Purchase orders for the General Electric XRD 6 unit and attachments were submitted immediately after we received notice of award of funds. Over the next several weeks the orders were processed

and submitted for bids. The bid was awarded in December 1962 and delivery was set for 24 February 1963. Parts of the XRD6 unit arrived on that date, but the main units did not arrive for another week. The engineer from General Electric, Mr. S. H. Stout, arrived on 19 March 1963 and began to assemble the machine. In the interim engineers at the base installed the necessary electrical and plumbing facilities. Installation of the machine was delayed by several unforseen events including hospitalization of Mr. Stout for a urethral stone and delays in obtaining certain cables and minor parts. By 20 May 1963 the major parts of the machine had been assembled and the remaining weeks of May and June were spent in checking the function of each item of equipment. We had expected to have the machine in operation by April or May, but it was not fully operative until late June.

During the period of acquisition and installation of the instrument, Doctors Earle and Johnson attended special courses in the latest methods of X-Ray spectrochemical analysis. Doctor Earle and Pfc. De Camp conducted experiments on sample preparation including fusion methods, extraction methods, liquid and powder preparation techniques. Pfc. De Camp was sent to the Norelco short course in X-Ray Spectroscopy in New York.

On 6 May 1963 Doctor I. Adler of the U. S. Geological Survey in Washington, D. C., kindly visited the project and gave us some helpful advice concerning instrumentation and sample preparation.

During the year additional samples of crystals from human pineal glands were extracted and diffraction patterns were obtained using the Siemens Crystalloflex II unit. These patterns were indexed and studied. Fluorescent spectrography studies will be carried out when the machine has been completed, checked and evaluated with known standards which have been ordered from the National Bureau of Standards.

Summary and Conclusions:

The initial period of this research project was spent largely in obtaining the General Electric XRD6 X-Ray Emission and Diffraction equipment, in installation, and in checking the component parts of the equipment. In addition, experiments were conducted in sample preparation and in powder diffraction patterns of crystals from human pineal glands using the Siemens Crystalloflex II equipment.

The X-Ray diffraction powder pattern of the crystals from twenty formalin fixed human pineal glands extracted with 1 N sodium hydroxide, revealed the pattern of an hydroxy apatite. We were concerned, however, by recent indications that the crystallinity may have been altered by the extraction with sodium hydroxide. Therefore, we plan to repeat the diffraction experiments using other methods of extraction of the crystals including blunt dissection. The diffractometer will be used to check the film diffraction pattern.

The long time required to obtain and to install the XRD6 unit has prevented us from conducting any definitive experiments in X-Ray fluorescent spectrography during the period of this report.

List of Publications:

None

ANNUAL PROGRESS REPORT

Title Page

PROJECT NO. 3A012501A802

TASK NO. 5

NAME AND ADDRESS OF REPORTING INSTALLATION:

Armed Forces Institute of Pathology

Washington 25, D.C.

NAME OF DEPARTMENTS AND DIVISIONS:

Department of Pathology

Military Environmental Pathology Division

Wound Ballistics Pathology Branch

PERIOD COVERED BY THIS REPORT:

7 January 1963 - 30 June 1963

PROFESSIONAL AUTHOR OF THIS REPORT:

Principal Investigator: Pierre A. Finck, Lt. Col., MC, USA

Assistants: None

REPORTS CONTROL SYMBOL: RCS-MEDDH-288

SECURITY CLASSIFICATION: Unclassified

ABSTRACT

PROJECT NO. 3A012501A802 TITLE: Combat Surgery
Wound Ballistics

TASK NO. 5

NAME AND ADDRESS OF REPORTING INSTALLATION:

Armed Forces Institute of Pathology

Washington 25, D. C.

PERIOD COVERED BY THIS REPORT:

7 January 1963 - 30 June 1963

AUTHOR: Pierre A. Finck, Lt. Col., MC, USA

REPORTS CONTROL SYMBOL: RCS-MEDDH-288

SECURITY CLASSIFICATION: Unclassified

Funds were made available to permit travel by Lt. Col. Pierre A. Finck, MC, USA and Lt. Col. Edward H. Johnston, MC, USA to London and Paris in connection with research of military interest.

Lt. Col. Finck presented a paper on "Nomenclature of Missile and Blast Wounds" at the International Meeting in Forensic Immunology, Medicine, Pathology and Toxicology in London, England on 16 April 1963. Lt. Col. Johnston also attended this meeting.

On 26 April 1963, in Paris, France, Lt. Col. Finck gave lectures at EUCOM Officers' Call on "Liaison between Medical and Criminal Investigation of Missile Wounds," and subsequently at the "Val de Grace" French Army Training Hospital on "Interpretation of Missile Wounds; Correlation between Ballistics and Injury. Correlation between American and Metric System Units Used in Ballistics." While in Paris, Lt. Col. Finck collected data and bibliographic references in the field of ballistics by visiting the Crime Laboratory of the French Police and by going to book-stores. A detailed trip report has been submitted to the Commanding General, Army Medical Research and Development Command.

In missile wound autopsy cases reviewed at the AFIP, action has been taken to improve research in the correlation between wounding agents and morphologic findings by requesting more information from contributors regarding the ballistic data of the cases such as:

A. Accident? Suicide? Homicide? Wounded in Action (WIA)? Died of Wounds (DOW)? Killed in Action (KIA)?

B. Type and Nationality of Weapon?

C. Caliber of Weapon? (In inches or in millemeters).

D. Name of Ammunition's Manufacturer?

E. Type of Bullet? (Shape; Lead, Coated, Jacketed).

F. Weight of Bullet or Fragment? (In grains or grams).

G. Impact Velocity of Projectile? (In feet per second or meters per second).

H. Impact Kinetic Energy of Projectile? (In foot-pounds or kilogrammeters).

I. Distance between weapon and victim?

J. Posture of victim at time of injury?

K. Degree of physical activity or incapacitation after injury?

L. Time interval between injury and death?

M. Time interval between death and autopsy?

ANNUAL PROGRESS REPORT

Project No.: 3A012501A806 - Internal Medicine

Task No.: 6 - INVESTIGATION OF RESPIRATORY DISEASES OF LABORATORY ANIMALS

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology, Washington 25, D. C.

Name of Department and Division:

Department of Pathology, Geographic Pathology Division, Virology Branch

Period Covered by the Report: 1 July 1962 - 30 June 1963

Professional Authors of the Report:

Principal Investigator: Erby L. Massie, Major, USAF, VC

Assistants: Jack D. Douglas, Major, USAF, VC and F. M. Garner, Major, USA, VC

Reports Control Symbol: RCS-MEDDH-288

Security Classification:

Unclassified

ABSTRACT

Project No. 3A012501A806 Title: Internal Medicine

Task No. 6 Title: Investigation of Respiratory Diseases of Laboratory Animals

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology, Washington 25, D. C.

Period Covered by the Report: 1 July 1962 - 30 June 1963

Authors: Erby L. Massie, Major, USAF, VC; Jack D. Douglas, Major, USAF, VC and F. M. Garner, Major, USA, VC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: Unclassified

Project No. 6X61-01-001-01 is devoted to a study of the epidemiology, diagnosis, treatment and prevention of respiratory diseases of laboratory animals. Respiratory diseases are responsible for great economical losses in animal colonies. This is true especially for the albino rat, the dog and the hamster. For example, experiments which use albino rats one year old or older run the risk of being invalid due to chronic murine pneumonia in the experimental animal. Our study, as well as studies by others, has shown that albino rats of all ages suffer from chronic pneumonia. Likewise, dogs purchased for research were found to be particularly susceptible to a respiratory disease similar epizootically to recruit fever in man. These dogs had to be cured before they were suitable for experimental use.

Last, hamsters with respiratory disease, like rats and dogs, are of little value as experimental animals until the disease is either controlled or eliminated.

The study supported by this project has done much to reveal the nature of the epidemiology of chronic murine pneumonia in the albino rat and in other animals. An agent has been isolated which is a PPLO. It is hoped that this will serve as a prototype for further studies with agents isolated from other animals. At the present, PPLO have also been isolated with great regularity from dogs suffering from respiratory infection. There is also serological evidence which strongly implicates this organism in the disease process. Also in one case respiratory disease was produced in the dog along with complement-fixation titer rise. At the present time about 100 different PPLO isolates have been made from rats, hamsters and dogs; and currently these isolates are being serologically typed. The cooperation of the Veterinary Section of WRAIR and the PPLO laboratory of the Department of Microbiology of WRAIR has been very helpful during the period covered by the report and their continuing support is anticipated.

BODY OF REPORT

Project No. 3A012501A806 Title: Internal Medicine

Task No. 6 Title: Investigation of Respiratory Diseases of Laboratory Animals

DESCRIPTION:

All types of domestic animals are used as experimental animals and we are rapidly tapping the resevoir of wild animals. Medical personnel think of experimental animals mainly as additional host systems for the study of infectious diseases. However, this is only one use for experimental animals. More and more, experimental animals are being use in psycology, physiology, nutrition, pharmacology, toxicology and radiology. The broadening peripheries of space exploration too call for more animals of all sizes and types to be used in any possible situation which might call for analysis of environmental factor conducive to human abode. Primary tissue for tissue culture must come from animals and only healthy, disease-free animals are satisfactory. It can be safely stated that the need for experimental animals has increased and will continue to do so.

Experimental animals must be free from infectious diseases or the experiment in which they are being used will be invalid. Nothing can add bias to an experiment faster than untimely deaths of the experimental animals or abnormal physiological reactions brought on by disease.

PROGRESS:

In the past we have worked with such diseases as feline rhinotracheitis, canine hepatitis, infectious bovine rhinotracheitis, canine distemper, chronic murine pneumonia and many other respiratory diseases relating to

the rabbit, hamster and the mouse. Currently, we are working with several possibly related diseases, for example murine pneumonia of the laboratory rat and kennel cough in the dog. The progress on work involving each of these diseases is given in detail in the following pages. Additional advancement has been made in several areas which, though not yet at a publishable state, open new avenues of approach to our studies involving respiratory diseases in laboratory animals and bring us closer to a more complete knowledge of the relationship of the agents involved.

A. Chronic Murine Pneumonia. Chronic murine pneumonia is a disease of the adult laboratory rat. The disease seldom occurs in animals under one year of age. The number of rat colonies affected with this disease is not known but our experience shows the incidence to be extremely high. The disease is very difficult to control and thus far has been impossible to eliminate.

Experiments are currently underway in our laboratory aimed at control of the disease through the use of antibiotics both in the food and the drinking water. Some of these experiments look promising. The best method we have found for control so far, however, has been good animal husbandry practices along with controlled humidity not exceeding 40%.

No bacteria have been isolated with frequency but on occasion streptococcus, Brucella and other bacteria have been found. PPLO are found in abundance and in practically every diseased lung examined. At least three different morphological types are present. To date, no virus has been isolated; however, efforts toward isolation will continue.

The most encouraging aspect of chronic murine pneumonia so far has been our ability to produce the disease in 10-gram albino mice, serially

pass the disease in mice, transmit it to young rats and back again to mice. To do this, we ground the diseased rat lung with four parts of a diluent consisting of saline and 10% dog or rabbit serum with penicillin 500 u per ml. The lung mixture must be kept at a temperature not to exceed 4°C. After the mixture is homogenized, it is centrifuged at 2,000 RPM for 30 minutes at 4°C. The supernate is then collected and kept stored at -70°C until ready for use. The homogenate supernates will keep for approximately 6 months at this temperature. Mice are inoculated intranasally with 0.05 ml of the homogenate. No other route of inoculation will produce the disease nor will the mice infect other mice, naturally, at any period following inoculation.

To date, the albino mouse is the only host system other than the rat. We have likewise been unable to establish the agent in any of a dozen or more tissue culture lines tried. Infectivity is lost if the homogenate is held overnight at 4°C. The agent in the infectious supernate can be further spun down at 8,000 gravities for 30 minutes. The agent will pass freely through an HA filter of pore size 0.45 u $\pm$ .02 u. It is resistant to penicillin and streptomycin.

The usual medium for the isolation of rat PPLO is Difco PPLO fortified with 10% horse serum and 5% fresh yeast extract. Primary isolation is easy but recovery of the organisms after the homogenate is introduced into the mice is difficult. We have solved this problem by abandoning the Difco agar in favor of a rat infusion broth. The infusion agar is made from one pound of ground rat muscle to one liter of distilled water. To this broth are added 14 gm of agar per liter for a PPLO agar or it is left as a broth

if desired. The medium has proven very satisfactory for isolation of rat PPLO. We now have several isolates on serial passage and we have recovered PPLO from mice following inoculation with a rat lung homogenate. These isolates will be used for the production of complement-fixation antigen and subsequent diagnosis of the disease in the rat.

B. Acute Respiratory Disease of the Dog. Dogs which are brought together in large numbers in kennels or animal colonies are susceptible to a disease called kennel cough. Not uncommonly, 50% of the animals will be infected and 25% will die. The mortality rate can be reduced considerably by good nursing and veterinary care. The exact etiological agent for the disease is not known. We became interested in the disease because of the great losses encountered in WRAMC Kennel. At first, screenings were made for possible atypical distemper, then for infectious canine hepatitis and other adeno type viruses, but none were found. We were able, however, to find large numbers of PPLO. One isolate was injected intravenously in a 6-month old pup and disease clinically identical to that seen in the kennel cough was produced. A complement-fixation antigen was made which showed no titer at disease onset to the same agent injected but did react in a 1/64 dilution 30 days after onset of the disease. The same antigen has been used to test dogs affected with the disease. To date, 125 paired dog sera from diseased dogs have been collected. Fifty paired sera have not been tested and are being held at -70°C. Of the remaining 75, 28 were suitable for testing and 47 blood sera were anticomplementary, either in the acute or convalescent sera or both. Of the 28 tested, 9 showed what could be considered a significant change in complement-fixation titer. This number of reactions is admittedly low but encouraging. Improvement of the antigen and better blood drawing technique should influence future results.

C. Respiratory Disease of Hamsters. Work is continuing on a limited scale with respiratory disease in hamsters. A PPLO agent has been isolated and passed. The disease seems to be similar to respiratory disease in the rat; however, the albino mouse does not seem to be affected as in the case of chronic murine pneumonia.

SUMMARY AND CONCLUSION:

This project is designed to study respiratory diseases of laboratory animals. Such a study is necessary as many respiratory diseases of laboratory animals are not well understood. The lack of knowledge of these diseases often invalidates experiments involving laboratory animals. The early part of this year was spent studying chronic murine pneumonia. PPLO have been isolated on numerous occasions from albino rats suffering from pneumonia. From these experiments it has been found that the agent is very heat-sensitive, filterable through a bacteriological filter and resistant to high concentrations of penicillin and streptomycin.

Later in the year much attention was directed toward respiratory disease in dogs. A PPLO has been isolated and at present seems related to the disease process. Disease has been produced with the agent and change in complement-fixation titer noted in animals suffering from respiratory disease. Remaining now is a period of more extensive survey to confirm what has been thus far demonstrated.

A modification of existing media for the cultivation of all PPLO has enabled the laboratory to more easily culture the micro-organism. The production of "B" and "F" antigen component from PPLO culture has opened the way for future serological study. This appears to be the key which will unlock the problem of respiratory disease not only in the dog but in the rat and hamster as well.

LIST OF PUBLICATIONS:

Papers Published July 1962 - June 1963

1. Stability of the Virus of Feline Viral Rhinotracheitis. Glen W. Miller and Robert A. Crandell. Am. J. Vet. Res. March 1962, 351-353.

2. Brucellosis in Man and Animals in Turkey. S. Alpar and E. L. Massie. Turk Veterinary Hekin. dern. Derg. March 1962, No. 32, 53-57.

Manuscripts Submitted for Publication

1. Cytochemical Studies of the Intranuclear Inclusion of Feline Viral Rhinotracheitis Virus. R. A. Crandell and D. F. Hersey. Am. J. Vet. Res.

Manuscripts in Preparation

1. Chronic Murine Pneumonia in the Albino Rat. E. L. Massie. Veterinary Medicine.

ANNUAL PROGRESS REPORT

Title Page

Project No. 3A012501A806. Communicable Diseases.

Task No. . 7 Preparation and Use of Specific Fluorescent Antibodies.

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology,
Washington 25, D. C.

Name of Division and Branch:

Geographic Pathology Division,
Immunology and Bacteriology Branch.

Period Covered by the Report: 1 July 1962 - 30 June 1963.

Professional Authors of the Report:

Principal Investigator:

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Chauncey W. Smith, Major, USAF, MSC

M. David Hoggan, Captain, MSC, USA

Reports Control Symbol: MEDEH-288.

Security Classification: Unclassified.

ABSTRACT

Project No. 3A012501A806. Title: Communicable Diseases.

Task No. 7 Title: Preparation and Use of Specific Fluorescent Antibodies.

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology
Washington 25, D. C.

Period Covered by the Report: 1 July 1962 - 30 June 1963.

Authors: Joseph F. Metzger, Lt Col, MC, USA

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Reports Control Symbol: MEDDH-288

Security Classification: Unclassified.

The rapid diagnosis of infectious disease agents by immunofluorescent, immune electron microscopy, and other serological techniques has been continued. The separation of viral particles of EAV from plasma by the agar column technique has made possible the production of specific immune sera without a protein menstruum response. Consideration is given to the part played by mutual enzymes in different organisms, in the production of antibodies which give cross

reactions. Characterization of various fractions of Equine Abortion Virus has been extended. The dual response of an antibody in immunofluorescence with and without complement has been described in a Listeria monocytogenes system. Serological studies regarding the antigens of the three species of Leishmania infecting man are presented.

BODY OF REPORT

Project No. 3A012501A806. Title: Communicable Diseases.

Task No. 7 Title: Preparation and Use of Specific Fluorescent Antibodies.

Description:

The global concept of military medicine has enlarged the scope of its application, so that it is necessary to investigate a large spectrum of infectious disease agents heretofore considered unnecessary. In an adverse situation, rapid identification is a necessity. An ideal approach is through such immunological means as immunofluorescence and immune electron microscopy, however the reliability of these techniques is dependent upon antisera of high specificity. The immune response of animals for the production of such antisera requires improvement in the antigens used for stimulation, to avoid production of antibodies which could give undesirable reactions. Therefore, it is always necessary to attempt new techniques to increase our knowledge concerning the antigens of infectious agents if progress is to be made. The studies reported have been accomplished with the anticipation that an improvement in the rapid diagnosis of infectious diseases can be effected and confidence in the results of the immunological tests will be realized.

Progress:

Production of Immune Sera. It has been previously reported that virus can be isolated from infected plasma by differential centrifugation. This technique was evaluated to provide a method of rapid diagnosis of viral infections. Also reported was the identification of virus in experimental tissues by immune electron microscopy. With the use of immunological tools of increased sensitivity, added emphasis must be placed on the specificity of the immune sera used. One major problem with the production of immune sera to viral agents has been the dual response, i.e., (1) to the viral agent and (2) to the contaminating protein menstruum containing the virus. It has been recently reported that plant virus and its contaminating menstruum can be separated by using granulated agar gel columns. Our approach in using this technique has been twofold: (1) the separation of animal virus from infected plasma and/or cellular extracts of tissue culture for rapid identification by immune electron microscopy; (2) purification of virus from its protein menstruum to increase the specificity (i.e., immune response to only the viral antigen).

The model system has been based on using a hamster-adapted strain of equine abortion virus (EAV). Golden hamsters were inoculated and at 18-24 hours, when the animals were moribund, heparinized plasma was collected.

Agar gel columns of varying diameters, column heights, agar concentrations, and flow rates were used. The particle size of the agar was constant; the agar was passed through graded screens, 10 through 50,,and agar that did not pass through 60-mesh screen was used. Phosphate-buffered saline, pH 7.2 (PBS) was used in all systems tested. The results reported were found to be most reproducible when using a 2 cm column containing 3% agar with an optimum height of 48-52 cm after washing and packing. The flow rate of the PBS was maintained at 15 ml/hr by a micropump. The Rf value of this column was found to be 65 ml, using the dilute India ink technique. The main criteria in the use of this column are the use of purified agar that has been thoroughly washed with PBS and a critical control of the flow rate.

Five ml of heparanized EAV plasma was introduced into the column. The effluent was collected in measured aliquots with a continuous recording of the ultraviolet spectrum. All procedures were at room temperature. After 65 ml of effluent had passed through the column there appeared a small peak which continued for about 30 ml, at which time a larger second peak appeared. Using rabbit anti-hamster serum against the aliquots from both peaks, in the precipitin reaction, it was found that there was no hamster protein in the first or smaller peak, however, protein was detected

throughout the second or larger peak. Using the Folin-Ciolcalteau test for protein, similar results were obtained.

Infectivity studies using aliquots from both peaks indicate that the virus had been separated from its protein menstruum, the major portion of the virus being in the first or smaller peak (virus peak). The major concentration of the virus was at the height of the virus peak. As compared with the original LD_{50} titer of the infected hamster plasma, there had been a loss in infectious virus. To determine if this loss in infectivity was due to temperature or UV inactivation, runs were made in the cold (4°C) and aliquots collected without UV scanning. When collected by this method there was no apparent loss in the infectivity of the virus.

Tests were made to determine the effect of this procedure on the complement-fixing activity (CF) of the virus. Numerous 50% end-point CF tests were made. The virus peak contained less than 10% of the CF activity of the unfractionated material. There was no apparent concentration of the CF activity except that the CF activity was constantly higher and uniformly distributed throughout the second or larger peak (protein eluate).

Employing the technique reported in the previous fiscal year, a cesium chloride gradient was used to ascertain the separation of the virus from its protein menstruum. The

fraction from the height of the virus peak contained one infectious band (1.271 g/cm^3) and a second non-infectious band (1.246 g/cm^3). A fraction at the lower end of the peak showed the same results, which were much less pronounced than in the previous fraction. In a fraction taken from the protein peak, no virus bands were in evidence, however, there appears to be a protein band.

Previous attempts to produce a specific immune serum to EAV in a burro gave an antiserum in which the response to the protein menstruum overshadowed the response to the viral agent. This was most evident when a booster immunization was employed to raise the viral titer. The fractions from the height of the viral peak were used to produce an immune serum in a burro.

Even when using a booster immunization to increase the viral titer there was no viral response to the protein menstruum by the CF test. There was produced an immune serum with a high degree of specificity for the viral agent without the problem of nonspecific immune responses to normal hamster plasma. Although many have used absorption techniques to attempt to rid immune sera of these undesirable responses, with the increased sensitivity of recent immunological tools it is not always possible to eliminate the immune response due to contaminating substances accompanying the viral agent

employed. This can result in a loss of confidence of specifically detecting a virus agent as well as cause not only a loss of time, but also up to fifty per cent of the sera in the process. The use of agar columns to purify EAV from contaminating proteins is both feasible and reproducible. With this simplified technique adequate viral antigen can be obtained rapidly to produce immune sera of a higher degree of specificity. Other methods of purification, such as high-speed centrifugation, ion-exchange columns, etc., can cause damage to the virus. With agar columns the treatment is bland in that the pH and molarity are constant and the passage of the virus in the agar does not appear to harm the virus.

EAV being a larger virus (120-180 mμ), preliminary experiments were repeated on a smaller virus, infectious canine hepatitis (ICH) (60-70 mμ). ICH was grown on Maden dog kidney cells. When maximum growth was in evidence the supernate was removed from cellular debris by centrifugation. The preliminary results indicate a separation of a viral and a protein peak, as previously described.

In attempting to produce specific immune sera for immunofluorescence and immune electron microscopy, various biophysical means have been applied for the production of specific antigens. This has been difficult; for example,

the separation of polysaccharides and mucopolysaccharide complexes of capsular material into their various specific antigens has not been completely effected. One must also consider the complex cell wall and the somatic antigens. One consideration which has not received a great deal of study is the possible cross-reactivity due to common enzymes in various agents. In investigating this possibility, immune sera have been produced to several common enzymes. Two of these, alkaline phosphatase and catalase, have been studied by immunofluorescence. Organisms tested, which are known catalase positive, gave a positive immune response. Using E. coli in a chemically defined deficient medium in which alkaline phosphatase is produced, the specific immune serum could detect the enzyme. Further studies are in progress to determine the role of mutual enzymes in cross reactions. Previous reports have indicated that there is a common antigen in gram positive organisms, the Rantz Factor. Further investigations will delineate whether these are due to common antigens or enzymes.

Virus Studies. Our studies on equine abortion virus have continued during the past year. At the time of our last report it was possible to separate crude virus material into three virus fractions, using a cesium chloride density gradient. Fraction E, which contained over 95% of the infectious virus, consisted of particles having double membranes and an over-all

diameter of 170-190 millimicrons. Fraction F, which contained very little infectious virus or complement-fixing antigen, did contain the bulk of virus-like material. This fraction consisted of irregular particles which had a diameter of 110-120 millimicrons. Fraction AB, which contained over 70% of the total complement-fixing activity, could not be pelleted and sectioned like the other material. However, during the past year, utilizing pseudo-replica techniques and shadowing with platinum palladium, it has been possible to visualize this complement-fixing material. It consists of small doughnut-shaped particles that have a diameter between 10 and 15 millimicrons. This material is believed to be part of the coat of the infectious particle which is formed in the cytoplasm during maturation. As described earlier in this report, adaptation of an agar gel column technique has made it possible to effect a good preliminary purification of the virus material with little loss in infectivity. It has been possible to separate the host contaminating protein and most of the complement-fixing antigens from the material which has been designated Fractions E and F. This material has proved to be excellent material for immunizing animals without the production of antibodies to the hamster host.

An additional model viral system is in the process of being investigated, murine hepatitis virus (MHV). This agent is commonly isolated from "normal" mouse colonies as well as in wild rodent populations. The host's nonspecific defense mechanisms, maternal antibody, and presence or absence of erythrozoan parasites greatly alter the effects and outcome of the infection. As this is a natural infection in mice it should present results different from the response of EAV in hamsters which is more of an unnatural system. The pathogenesis of the disease will be correlated with the immune response (i.e., immunofluorescence, CF activity, and immune electron microscopy, etc.) as previously used in the study of EAV in hamsters.

All strains used have produced a fatal disease in suckling mice in 96 - 120 hours. Specific immune serums to the virus strains have been produced in rabbits and mice. By immunofluorescence an immune response can be observed in the liver 36 - 48 hours after infection, usually in areas of primitive hemopoietic cells. No gross changes are in evidence and histopathological changes are so subtle as to be almost undetectable. However, by 72 hours focal tuberculoid type lesions can be observed grossly. The histopathological changes are very apparent, involving all cellular elements of the liver. The concentration of infectious virus and

complement-fixing antigens begins to increase at about 36 - 48 hours and continues to 72 hours. Thereafter it remains relatively stable until death.

The immunological relationships of the various strains used are also being studied by gel diffusion plates. It is interesting to note that there is a cross reaction between two of the strains by immunofluorescence and complement fixation which cannot be shown by immune electron microscopy. Further studies of antigenic relationships and pathogenicity variations of the strains are being made.

Immunofluorescence. The rapid identification of all infectious disease agents is a continuing process. As outlined in the previous progress report, investigations are in progress to produce large pools of immune sera to provide sufficient screened reagents for field testing. The animal of choice for production of large amounts of antiserum has been the burro. The immune response of the burro has been found to be most divergent in many instances from that of other animals. Specific immune serum is being produced and it is anticipated that in the next few months sufficient serum will be available for field testing.

There has never been a direct relationship between the normal serological reactions (i.e., precipitin, agglutination, complement fixation, hemagglutination, etc.) and immunofluorescence. On many occasions it has been reported in the literature that a certain immune serum had given a

specific immunofluorescent response and elicited a certain serological response. There was no evidence that the serological titer and the immunofluorescent response were related. In our investigations it has been found that normal serological titers cannot be related to a significant immunofluorescent response. This leads one to wonder concerning the immune reactions that are involved. Using as a model Listeria monocytogenes, some very interesting results have been obtained as detected by immunofluorescence. Many immune sera were prepared by several methods. These sera were divided into two groups - positive and negative immunofluorescent results. All sera when tested for complement-fixing activity gave a significant titer. All sera were tested by mixing fresh guinea pig complement with the conjugated sera. The sera that had given an immunofluorescent response now gave a more intense response. The sera that had not given an immunofluorescent response now gave a response. Adequate complement, normal and immune sera controls were used to determine that the reactions were specific. These observations are of importance when small amounts of antigen are present. It appears that a dual serological response can be utilized with immunofluorescence - the normal immunofluorescent response and in this case the complement fixation response. The dual response may make it easier to observe the immune reaction and due to added intensity will increase the period of fluorescence.

Immune Electron Microscopy. With immune electron microscopy (IEM) it is also very important to use conventional techniques to correlate the architecture with that observed by formalin fixation and the immune reaction. In the separation of virus from its protein material by the agar column technique all investigations were monitored by observing the aliquots to determine the location of the viral particles and their structure. The technique of particle counting by depositing virus on agar with high-speed centrifugation has been investigated. The technique appears to have possibilities on placing virus in an efficient pattern on a grid. By this method a specific immune serum could be applied for rapid identification.

As previously outlined, enzymes can produce immune responses. In preliminary investigations using E. coli in a specially defined medium, it was possible to observe the site of alkaline phosphatase activity by an adapted histochemical procedure. Future experiments will be made to correlate these sites by an immune reaction.

Using the tetrazotized benzidine technique of conjugating ferritin with an immune serum the specificity has been confirmed. Whole organisms of N. meningitidis were treated with specific labeled immune serum and the immune reaction could be observed in the capsular portion of the organism.

The true role of IEM is yet to be described. Some applications could be: (1) To be used in checking the specificity in the production of specific immune reactions (capsular, cell wall, and somatic); to further determine if antigens are diffuse in a specific area, or are in a defined area. (2) To study the pathogenesis of infectious diseases to determine the role of the immune reaction in tissue destruction as well as the localization of various infectious processes. (3) To identify virus by a specific immune reaction in biopsy material. Undoubtedly there are often many innocuous viruses present which have no bearing on the infectious process. IEM could delineate this problem. By conventional techniques of electron microscopy they could not be differentiated.

Vector-Borne Diseases. The proclivity of United States fighting men for infections ever present in tropical and subtropical areas was amply illustrated during World War II. Then American fighting men were exposed in numbers as never before to malaria, schistosomiasis, and Kala Azar. In any future world-wide hostility it is very probable that they will be exposed to Leishmaniasis and Trypanosomiasis. Thus, it is important to ascertain the best methods for rapid identification of the etiological agent involved.

Leishmaniasis. The identification of the three species of Leishmania usually isolated from man has been determined in the past on the basis of clinical findings in the patient and the geographical area of origin involved. It has been suggested that these agents are all the same organism, as their morphology indicates, and that the clinical differences are due to passage through the various species of Phlebotomus involved as vectors.

It is apparent that a classification based upon the antigenic differences of the three species, L. donovani, L. tropica, and L. braziliensis, is essential before further investigative studies can be properly oriented.

After preliminary studies had indicated that L. tropica was probably antigenicly different from L. donovani and L. braziliensis, cultures of additional strains of the latter two species were obtained in South America (see Annual Progress Report, 1 July 1961 - 30 June 1962). Two additional strains of L. tropica were obtained from Dr. P. E. C. Manson-Bahr during last December 1962. Saline-washed cultures of these additional strains have been lyophilized for use in serological tests and satisfactory antisera have been prepared for most of the strains.

The antigen-antibody relationship of the various strains is presently being studied by the Ouchterlony test. Studies

to date indicate that rabbit antisera prepared against the Khartoum strain and two strains of L. donovani isolated from cases of Kala Azar in Brazil give similar precipitation lines when tested against antigen prepared from Khartoum strain organisms. Similar tests employing strains of L. braziliensis obtained from Costa Rica, Venezuela, and Brazil have failed to demonstrate common precipitation lines. The antiserum prepared from a Venezuelan strain which has been designated as L. braziliensis pifanoi gives precipitation lines which demonstrate a closer relationship to strains of L. tropica from Africa. One strain from northern Brazil appears to be similar to L. braziliensis pifanoi, but further studies are required.

Thus it appears at this time in this preliminary report that there are actually Leishmania braziliensis and Leishmania tropica strains in South America causing cutaneous leishmaniasis. Also these studies at this time would seem to indicate that Leishmania donovani, causing Kala Azar, is antigenicly similar in Africa and South America.

Trypanosomiasis. Work has continued on the attempt to chemically isolate the antigenic fraction or fractions of Trypanosoma cruzi which will give a sensitive result in the complement fixation test for Chagas' Disease, without the nonspecific reactions encountered with some syphilitic and normal sera. It has been possible by controlling various

physical and chemical factors to isolate a protein which gives sensitive results and with which there has been a reduction in the number of nonspecific reactions. Further purification of this protein is being attempted.

A conjugated antiserum, prepared against the whole organism, will produce immunofluorescence of T. cruzi organisms in tissue from experimentally infected animals. However, no tests have been made to determine the specificity of this serum. If antiserum can be produced that is specific, it should be of value in the diagnosis of cases which come to autopsy with gross and microscopic pathology which is compatible with Chagas' Disease. T. cruzi organisms are seldom found in sections of heart from such cases, so at present the diagnosis must be based on the gross and microscopic findings with due consideration of the medical history and the area of residence

List of Publications:

Smith, C. W., Metzger, J. F., and Hoggan, M. D. Immunofluorescence as applied to pathology. Am. J. Clin. Path. 38: 26-42, 1962.

Metzger, J. F., Kase, A., and Smith, C. W. Identification of pathogenic fungi in surgical and autopsy specimens by immunofluorescence. Mycopathologia et Mycologia Applicata XVII: 335-344, 1962.

Smith, C. W., and Metzger, J. F. Demonstration of a capsular structure on Listeria monocytogenes. Pathologia et Microbiologia 25: 499-506, 1962.

Metzger, J. F., and Smith, C. W. Serologic typing of Listeria monocytogenes. Proc. Soc. Exper. Biol. Med. 110: 903-906, 1962.

Metzger, J. F., and Smith, C. W. The application of immune electron microscopy to the demonstration of antigenic sites in biological systems. Lab. Invest. 11: 902-911, 1962.

Smith, C. W., and Metzger, J. F. The dynamics of the serology of Listeria monocytogenes. Proceedings of Second Listeric Conference, 1962.

Smith, C. W., and Metzger, J. F. Identification of Listeria monocytogenes in experimentally infected animal tissue by immunofluorescence. Proceedings

of Second Listeric Conference, 1962.

Zachs, S. I., Metzger, J. F., Smith, C. W., and Blumberg, J. M. Localization of ferritin-labelled Botulinus toxin in the neuromuscular junction of the mouse. J. Neuropath & Exp. Neurology. XXI: 610-633, 1962.

Metzger, J. F., Smith, C. W., and Hoggan, M. D. Agar column purification of virus. I. Purification of equine abortion virus from infected hamster plasma. In press.

ANNUAL PROGRESS REPORT

Title Page

Project No. 3.0125C1A303) - Internal Medicine Metabolism and Nutrition

Task No. . (Radiation and Sterilization of Foods)

Name & Address of Reporting Installation:

(Armed Forces Institute of Pathology, Washington 25, D. C.)

Name of Department and Division:

(Department of Pathology, Veterinary Division)

Period Covered by the Report: (1 July 1962 - 30 June 1963)

Professional Authors of the Report:

Principal Investigator: Lt. Colonel M. A. Ross, VC

Assistant: Major F. M. Garner, VC

Reports Control Symbol: (RCS-MEDDH-288)

Security Classification:

(Unclassified)

ABSTRACT

Project No. 3A012501A803 Title: Internal Medicine Metabolism and Nutrition

Task No. 8 Title: Radiation and Sterilization of Foods

Name and Address of Reporting Installation: Armed Forces Institute of Pathology
Washington 25, D. C.

Period Covered by the Report: 1 July 1962 - 30 June 1963

Authors: Lt. Col. M. A. Ross; Maj. F. M. Garner, VC

Reports Control Symbol: (RCS-MEDDH-288)

Security Classification: (Unclassified)

SUMMARY

All of the material from the initial long term feeding contracts using rats, dogs and monkeys as the experimental animals has been received. The material included parent generations of all species plus material of succeeding generations in rats and dogs. The number of cases received, reviewed, coded and put on IBM cards are rats 2942, dogs 291, and monkeys 39 for a total of 3272 cases. Several hundred cases from extra or special feeding groups that have been received and accessioned will not be included in the individual contractor reports. Material from two current projects are being received and a decision to process or file only will be made at a later date.

Abstract - cont'd

Occasional differences are still being found that are significant at the 10% level. There is however, no clear nor consistent pattern observed that would suggest that the factor of food irradiation at either level used was responsible. The differences were frequently due to a higher incidence of lesions in the controls or with sex. A summary report on thyroiditis in dogs was submitted. No evidence could be found to suggest that food irradiation was responsible.

BODY OF REPORT

Project No. 3A012501A803. Title: Internal Medicine, Metabolism and Nutrition; Radiation and Sterilization of Foods

Description: This is a continuation of the program developed by the R & D Division of the Office of the Army Surgeon General designed to produce experimental data relating to the possible toxicity of foods sterilized by irradiation. Seventeen contractors fed irradiated foods to rats, dogs, or monkeys for two-year periods. Animals that died during the course of the study or that were sacrificed at the termination of the feeding period were autopsied. Tissues were harvested from each animal, processed and examined histologically. The paraffin blocks, a duplicate set of slides and a complete protocol, including the histopathological findings for each animal, were forwarded to the Armed Forces Institute of Pathology for file, review and IBM coding.

All of the material anticipated has now been received. Additional material derived from special studies being conducted by the Bowman Gray Medical School and Virginia Polytechnic Institute is being received. A firm decision to process these cases similarly to previous materials is being considered.

Contract reports for the following programs were completed during the year and submitted as portions of the semiannual progress reports required by the project.

Contractor	Species	Test Food(s)
Cornell University	Dog	Potato
MIT	Dog	Eggs
Vanderbilt	Dog	Beef, Jam, Chicken
Oregon State University	Rats	Pork, Carrot
Syracuse University	Rats	Whole Orange

The following contract reports are now in various stages of preparation and it is anticipated that they will be submitted either with the next semiannual progress report due in September 1963 or in March 1964.

Contractor	Species	Test Food(s)
Cornell University	Dog	Chicken, Beef, Pork
Vanderbilt University	Monkey	Peach, Whole Orange, Peeled Orange
Oregon State University	Rat	Jam and Flour
Syracuse University	Rat	Shrimp and orange, Chicken and cole slaw
Alabama University	Rat	Sweet potato and codfish
University of Miami	Rat	Evaporated milk
Vanderbilt University	Rat	Beef
Hazelton Labs.	Rat	Corn and tuna

Progress in the preparation of the above contract reports during this fiscal year was considerably reduced due to resignations and protracted illnesses of principal and assistant investigators. The statistical workups however, have progressed satisfactorily and essentially all contracts are either completed or in progress and awaiting further work by the principal investigators.

There has been no pattern observed that would suggest that the factor of food irradiation, irrespective of level, was a causative factor in the production of histologic change. The 10% level of significance was intentionally chosen to point out those areas in which differences were present. Many of the differences would not have been significant had the level chosen been 5% rather than 10%. The controls were frequently more affected than the animals of the irradiated diet groups although the most significant differences has occurred between the sexes.

Summary and Conclusions:

All of the material from the original contractors feeding irradiated

foods to rats, dogs or monkeys for a period of two years has been received. A sizeable group of contract reports are expected to be completed for submission with the semiannual progress report due in September 1963 and March 1964.

Though differences of significance at the 10% level are still being noted, there has been no consistent pattern. Sex differences are most often noted as are sporadic instances wherein either controls or intermediate diet groups are responsible for the differences. Therefore, no conclusions to the effect that consumption of irradiated foods by rats, dogs or monkeys, can be made.

The original intention was to consolidate into a single overall report, all material received by each species. However, with clearance for each food item being requested individually, the necessity for such an overall evaluation is questionable especially for rats. This is particularly true inasmuch as some doubt would exist as to the validity of statistical conclusions drawn from heterogenous strains since such studies would have diet and environmental differences in addition to those of strain. A formal request for an opinion in this matter will be forwarded to proper authorities at an early date.

List of Publications:

Presentations:

Several presentations were scheduled but not given because of illnesses. Information had been derived from Irradiated Foods Material.

Publications:

None.

ANNUAL PROGRESS REPORT

TITLE PAGE

Project No. 3A012501A806	Preventive Medicine
Task:	Immunization
Subtask No. 9	Specificity of the Immunologic Response To Chemical Protein Conjugates

Name & Address of Reporting Installation:

Armed Forces Institute of Pathology

Washington 25, D. C.

Name of Department:	Immunobiology
Period Covered by Report:	1 July 1962 to 30 June 1963
Professional Authors of Report:	
Principal Investigator-	Arthur M. Silverstein, Ph. D.
Collaborator-	Felix Borek, Ph. D.
Reports Control symbol:	MEDDH-288
Security Classification:	Unclassified

ABSTRACT

Project No. 3A012501A806 Title: Preventive Medicine

Task: Title: Immunization

Subtask No. 9 Title: Specificity of the Immunologic Response to Chemical Protein Conjugates

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology

Washington 25, D. C.

Name of Department: Immunobiology

Period Covered by Report: 1 July 1962 to 30 June 1963

Professional Authors of Report: Arthur M. Silverstein, Ph. D.

Felix Borek, Ph. D.

Security Classification: Unclassified

This report describes a series of studies on the specificity of delayed hypersensitivity reactions, aimed at improving our understanding of the mechanisms involved in this phenomenon and at clarifying its relationship to antibody mediated hypersensitivity reactions. It was found that: 1) saccharides of various types are able to function in the delayed system as haptenic determinants just as effectively as are ionic and nonpolar chemicals; 2) the delayed responses to haptens with side chains of varying length showed cross-reactions typical of those expected from the study

of other hapten systems; 3) the earliest antibody produced by the guinea pig following delayed hypersensitivity was found to possess the broad specificity typical of the delayed system rather than the narrow specificity usually found in circulating antibodies; 4) using radio-iodine labeled antigen, it was demonstrated that delayed skin reactions could be elicited in the presence of appreciable concentrations of circulating antigen. The latter observation is especially important since it argues against the postulated participation of highly avid circulating antibodies in the delayed hypersensitivity reaction.

BODY OF REPORT

Project No. 3A012501A806	Preventive Medicine
Task:	Immunization
Subtask:	Specificity of the Immunologic Response to Chemical Protein Conjugates

Description:

The objectives of this investigation are: 1) to study the immunological specificities involved in the delayed hypersensitivity reaction and to compare them with the specificities of antibody mediated reactions; 2) to study the nature of delayed inflammatory immunization reactions which may be induced by homologous and cross-reacting chemical-protein conjugates. These studies will test the hypothesis that there exists a fundamental difference between the mechanisms of delayed and immediate hypersensitivities, and that there may be a close relationship between delayed cellular reactions and the act of antibody formation.

Progress:

A. Delayed hypersensitivity to saccharide-protein conjugates. Delayed hypersensitivity was induced in guinea pigs to p-diazophenylglucoside or o-diazophenylgalactopyranoside coupled to guinea pig albumin (gpA). In each instance, the homologous

conjugate elicited a typical delayed inflammatory skin reaction after intradermal challenge, while the conjugate of the heterologous hapten showed a distinct cross-reaction. Similar results were obtained with a p-diazophenyllactoside-gpA conjugate. Selective desensitization with either homologous or cross-reacting gpA conjugates demonstrated that the specificity of the delayed response was directed in part against the saccharide moiety. Skin tests with homologous haptens coupled to the unrelated protein carrier ovalbumin (oval) failed to cross-react, indicating that this specificity also involves a portion of the carrier protein, confirming other studies. Comparable results were also obtained with conjugates of glucuronic acid or galacturonic acid, coupled directly to the amino groups of gpA.

Thus it has been shown that, contrary to the prediction of some workers, monosaccharides and disaccharides can serve as haptenic determinants in the delayed system.

B. Delayed hypersensitivity to haptens containing a side-chain of varying length. The purpose of this study has been to find out the extent of cross-reactions in the delayed system of gpA conjugates of p-nitroanilic acids containing side chains with 2 to 8 carbon atoms. Guinea pigs sensitized with certain members of this series were skin-tested with all 7 members. The extent of cross-reactions varied with the difference in the length of

side-chain between the sensitizing and the test hapten. The results were consistent with those obtained previously by Silverstein and Gell (J. Exp. Med. 115, 1053, 1962) using Haptens differing by the position of benzene-ring substituents.

C. Comparison of specificity of the delayed reaction with that of the early antibodies in the guinea pig. Gell and Silverstein made a preliminary observation (unpublished) that the specificity of guinea pig antibodies produced in response to a single antigen injection changes over a period of several days following the first appearance of circulating antibody. While studying the specificity of delayed response to p-aminobenzoic acid-gpA conjugates prepared by using either diazonium or isothiocyanate derivatives of aminobenzoic acid, we found that the carrier and link specificites characteristic of the delayed reaction persist into the early, Arthus-type reactions which appear on the 12-14th day after the sensitizing injection. In order to study further the specificity of the early circulating antibodies, guinea pigs sensitized to either gpa-azobenzoate or gpA-NCS-benzoate were bled daily starting on the 7th day after sensitization. These sera were tested for antibodies by passive cutaneous anaphylaxis using homologous antigens and antigens heterologous with respect to either the hapten-protein

linkage (azo vs. NCS) or to the carrier protein (gpA vs. oval). It was found that the earliest antibody to appear (usually on the 10th - 11th day) was carrier and link specific; 1 to 6 days later depending upon individual variation, it was followed by the appearance of antibody which cross-reacted with antigens containing a heterologous link or carrier. Similar results were obtained with conjugates containing derivatives of p-nitroaniline as haptens. Treatment of the sera with 2-mercaptoethanol, a substance known to inactivate 19S macroglobulin antibodies which frequently appear early in the course of immunization, resulted in the inactivation of the earliest, carrier and link-specific antibody. We are presently attempting to determine what, if any, relationship exists between the molecular weight of the antibody and its specificity.

D. Demonstration of the persistence of delayed hypersensitivity in the presence of an excess circulating antigen. Karush and Eisen (Science 136, 1032, 1962) postulated that delayed hypersensitivity is mediated by a special type of highly avid circulating antibody, present in the serum in an amount too small to be detected by the existing methods (10^{-10} M). If this were the case, one would expect that the presence of circulating antigen in concentrations of about 10^{-8} M should neutralize this

avid antibody and therefore should completely abolish delayed skin reactivity. In order to test this hypothesis, guinea pigs were sensitized to human serum albumin (HSA). After obtaining intense skin reactions on the 8 day, HSA labeled with radioactive I^{125} was injected intravenously at doses of 0.5 mg. and 0.25 mg. This resulted in only a partial desensitization of the subsequent skin tests, the extent of desensitization being dependent on the desensitizing dose. An examination of the serum of the partly desensitized animals in a liquid scintillation counter showed the presence of labeled antigen at the concentration of the order of 10^{-8} M. Examination of sera from subsequent bleedings showed that the immune elimination of labeled HSA started after the 11th day following sensitization. These results argue against the possibility that delayed hypersensitivity is mediated by an avid circulating antibody of the type postulated, and support the notion that the mechanism of delayed hypersensitivity involves the activity of sensitized cells.

Summary and Conclusions:

1. Saccharides have been shown to function just as effectively in the delayed hypersensitivity system as other simple chemical hapten determinants.

2. The knowledge of the specificity and cross-reactions of the delayed system has been extended by a study of a homologous

series of nitro-anilic acid haptens of varying chain length. The results with these haptens are in general agreement with those obtained in other hapten systems.

3. The earliest antibody formed in delayed hypersensitive guinea pigs has been found to have a carrier-specificity typical of the delayed system. Only after several days does the more conventional hapten-specific antibody appear. These differences may, from preliminary observations, be due to two different antibodies, one a 19S and the other a 7S g-globulin.

4. Using radio-iodine labeled antigen, a partial persistence of delayed skin reactivity could be shown in the presence of concentrations of circulating antigen in excess of 10^{-6} M. These data argue strongly against the participation of a hypothetical avid antibody in the mechanism of delayed hypersensitivity.

List of Publications:

1) F. Borek and A. M. Silverstein, Delayed Hypersensitivity to Saccharide-Protein Conjugates, Federation Proc. 22, No. 2, 2729, 1963 (Abstract)

2). F. Borek, A. M. Silverstein and P.G.H. Gell, Delayed Hypersensitivity to Hapten-Protein Conjugates. III. Saccharides as Haptens. (In preparation)

ANNUAL PROGRESS REPORT

TITLE PAGE

Project No. 3A012501A806	Preventive Medicine
Task:	Immunization
Subtask No. 10	Fetal Response to Immunization

Name & Address of Reporting Installation:

Armed Forces Institute of Pathology

Washington 25, D. C.

Name of Department:	Immunobiology
Period Covered by Report:	1 July 1962 to 30 June 1963

Professional Authors of the Report:

Principal Investigator-	Arthur M. Silverstein, Ph. D.
Collaborators-	Keith L. Kraner, Capt., USAF(VC)
	Robert A. Prendergast, Capt. MC, USAR
Reports Control Symbol:	MEDH-288
Security Classification:	Unclassified

ABSTRACT

Project No. 3A012501A806	Title: Preventive Medicine
Task:	Title: Immunization
Subtask: No. 10	Title: Fetal Response To Immunization

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology

Washington 25, D. C.

Period Covered by Report:	1 July 1962 to 30 June 1963
Authors:	Arthur M. Silverstein, Ph. D. Keith L. Kraner, Capt., USAF, (VC) Robert A. Prendergast, CAPT., MC, USAR
Security Classification:	Unclassified

Fetal lambs were stimulated in utero with a variety of immunizing antigens, with skin homografts, and with non-specific lymphoid stimuli, and their serologic and morphologic responses studied at intervals thereafter. The data indicate that: 1) the fetal lamb is able even earlier than mid-gestation to form antibodies; 2) the earliest antibody response is entirely composed of beta-2M 19S macroglobulins, followed only later by normal 7S gamma globulins; 3) immunogenesis in the fetal lamb is not a

discrete event, but rather the lamb matures its ability to form antibodies to different antigens at different gestational ages; 4) the fetal lamb can produce appreciable amounts of gamma globulin apparently lacking in antibody function; 5) the fetal lamb can reject, from mid-term onward, skin homografts as competently as the adult; 6) homograft rejection in the fetal lamb seems from preliminary experiments to be unassociated with antibody activity.

BODY OF REPORT

Project No. 3A012501A806 Title: Preventive Medicine

Task: Immunization

Subtask: No. 10 Fetal Response to Immunization

Description:

It is important in understanding the response to immunization to possess a knowledge about the ontogenesis of the immune response --about its time of appearance in the developing fetus, the morphologic changes in the lymphoid tissue that accompany the immune response, and the cellular and molecular bases for this response. The present report describes investigations on the nature of the response in the fetal lamb to a variety of stimuli, specific and nonspecific, performed at different stages of gestational development. Advantage has been taken of the development of surgical procedures permitting a variety of intra-fetal procedures without interruption of pregnancy, including injection of the fetus, orthotopic skin grafting, and the collection of blood or tissue for study.

Progress:

A. Antibody production by the fetal lamb. Fetal lambs were injected intramuscularly with a variety of antigens, including

bacteriophage Phi X 174, horse ferritin, ovalbumin, diphtheria toxoid, killed Salmonella typhosa, and BCG, either in Freund's adjuvant or in saline. The earliest lambs immunized, at 60 days gestation, were able to make anti-phage and anti-ferritin antibodies within 6-10 days, but no antibodies to any of the other antigens were produced at this fetal age. Only at about 120 days gestation (normal term in the ovine is 150 days) did the lambs commence anti-ovalbumin formation. Anti-diphtheria toxin, antisalmonella, and anti BCG antibodies were not found at any time during detal life. Antibody production started in response to these antigens only some weeks or months after birth. Once antibody production against a given antigen was instituted, the titers in general increased with the duration of the stimulus. These data (taken together with the skin homograft results below) suggest that the fetus does not mature its immunologic abilities all at once, but rather in a stepwise fashion, dependent upon some unknown attributes of the antigens employed.

B. The relationship of antibody to globulin response. The earliest antibody response to bacteriophage is composed of 19S beta-2M globulin, whether or not adjuvant is employed for immunization. Only many weeks later is 7S gamma globulin antibody production seen. This accords well with observations on the initial antibody response made in other species.

In the absence of adjuvant, 7S gamma globulin production is absent or very limited. In response to adjuvant, the marked lymphadenopathy in the draining lymph nodes is accompanied by the production of relatively large amounts of 7S gamma globulin, most or all of which is without demonstrable antibody function. It would thus appear that the fetal lamb under certain conditions may produce a "normal" non-antibody gamma globulin - a point of considerable theoretical importance.

C. Homograft rejection by the fetal lamb. We have succeeded in verifying extensively the observation of Schinkel and Ferguson that the fetal lamb can reject a skin homograft. These observations have been extended to show: 1) that fetal rejection is as rapid and apparently as competent as that of the adult; 2) that the ability to reject a homograft appears at about 80-85 days of gestation; 3) that rejection in the fetus is unaccompanied by plasmacytosis either in the graft bed or in the draining node; 4) that rejection in the fetus is unaccompanied by detectable gamma globulin formation; and 5) that the fetus appears in preliminary tests to be able to reject a graft normally even in the presence of circulating rabbit anti-7S gamma globulin and anti-beta-2M globulin. All of these observations suggest that circulating antibody may not be an obligatory participant in homograft rejection. This point is of prime

importance in understanding the graft rejection phenomenon, and has not to our knowledge been satisfactorily testable with other experimental models.

D. Other Approaches. Experiments have been initiated along other lines, the results of which are still being analyzed. These include attempts at inducing immunologic tolerance in the fetal lamb, studies of the effect of non-antigenic stimuli on the lymphoid system and on protein production, and studies on the possibility of raising the level of response to an antigenic stimulus by pre-maturing the fetal lymphoid system with non-specific stimuli. The results of these investigations will be included in the future reports.

Summary and Conclusions:

1. Technics have been developed allowing such procedures as immunization or skin grafting of the fetal lamb in utero.

2. The fetal lamb is capable of initiating antibody formation earlier than the middle of gestation. This response is accompanied by a conspicuous lymphoid hyperplasia and the maturation of immunologically competent cells.

3. The development of immunological abilities by the fetal lamb is not a temporally unique event, but rather occurs in a step-wise fashion dependant somehow on the nature of the antigen.

4. The earliest antibody response is a 19S beta-2M macroglobulin, followed only much later by 7S gamma globulin antibodies.

5. The fetal lamb appears able to respond to adjuvant injections with the formation of appreciable amounts of 7S gamma globulin apparently lacking in antibody function.

List of Publications:

1. Congenital Syphilis and the Timing of Immunogenesis in the Human Foetus. Nature, 1962, 194,196

2. Fetal Response to Antigenic Stimulus. I. Plasmacellular and Lymphoid Reactions in the Human Fetus to Intrauterine Infections, Lab. Invest, 1962, 11,918.

3. Fetal Response to Antigenic Stimulus. II. Antibody Production by the Fetal Lamb, J. Exp. Med., 1963.

4. Fetal Response to Antigenic Stimulus III. Gamma Globulin Production in the Normal and Stimulated Fetal Lamb. J. Immunol., 1963, in press.

5. Homograft Rejection by the Fetal Lamb. In preparation.

ANNUAL PROGRESS REPORT

Title Page

PROJECT NO. 3A012501B813 - Basic Research in Life Sciences-Radiobiology

TASK NO. 11 Biological and Biochemical Effects of Microwaves

NAME AND ADDRESS OF REPORTING INSTALLATION:

Armed Forces Institute of Pathology
Washington 25, D. C.

NAME OF DEPARTMENT:

Office of the Scientific Director

PERIOD COVERED BY THIS REPORT: 1 July 1962 - 30 June 1963

AUTHORS: Robert E. Stowell, M.D.
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Vaman S. Waravdekar, Ph.D.

REPORTS CONTROL SYMBOL: RCS-MEDDH-283

SECURITY CLASSIFICATION: Unclassified

ABSTRACT

PROJECT NO. 3A012501B813 TITLE: Basic Research in Life Sciences-Radiobiology

TASK NO. 11 TITLE: Biological and Biochemical Effects of Microwaves

NAME AND ADDRESS OF REPORTING INSTALLATION:

Armed Forces Institute of Pathology
Washington 25, D. C.

PERIOD COVERED BY THE REPORT: 1 July 1962 - 30 June 1963

AUTHORS: Robert E. Stowell, M.D.
Peter J. Goldblatt, Capt., USAF, MC
Shiro Takashima, Ph.D.
Benjamin F. Trump, Capt., MC, USA
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REPORTS CONTROL SYMBOL: RCS-MEDDH-288

SECURITY CLASSIFICATION: Unclassified

SUMMARY

This research has been directed during the past year toward exploring the non-thermal and the thermal effects of electromagnetic energy on two types of systems: (1) macromolecules of biological significance and (2) unicellular organisms and cells in tissue culture.

(1) Studies on Macromolecules - a) Athermal - No non-thermal effects of high frequency electromagnetic energy on the structure and enzymatic activity of several macromolecules have been found;

alterations in the structure of deoxyribonucleic acid (DNA) had been observed with audio-frequency electromagnetic energy which, however, were shown to result from a D. C. component. b) Thermal Studies - High power field has been used for the step heating of DNA solutions in order to study the kinetics of the heat denaturation.

(2) Studies on unicellular organisms - When exposed to high frequency electromagnetic fields, several types of unicellular organisms have been found to exhibit abnormal motility phenomena; at the present time, we are attempting to prove that these effects are not related to thermal phenomena.

BODY OF REPORT

PROJECT NO. 3A012501B813 Basic Research in Life Sciences-Radiobiology

TASK NO. 11 Biological and Biochemical Effects of Microwaves

DESCRIPTION:

The general long term objectives of this research are:

(1) To study the effects of electromagnetic radiation on macromolecules.

(2) To examine the effects of electromagnetic radiation on biological systems.

(3) To explore the possible applications of microwaves as a tool in biological research.

(4) To seek fundamental information of potential ultimate clinical significance in consideration of health hazards to personnel working in the environs of megawattage radar (microwave equipment).

PROGRESS:

A. Background Information.

The absorption of electromagnetic energy in solutions of electrolytes, body fluids and mammalian tissues has been well documented throughout the rf and microwave spectrum. This absorption is capable of producing substantial thermal effects which are known to alter macromolecules and living cells. A more interesting possibility,

however, is that electromagnetic waves in this region may have non-thermal effects. The only generally accepted non-thermal effect at the present time is the so-called "pearl-chain" phenomenon which refers to the alignment of particles in suspension under the influence of an electromagnetic field.[2,3] This phenomenon has been given a theoretical treatment by Saito and Schwan.[3] There are scattered reports in the literature of non-thermal effects on macromolecules.[1,2,6] In 1946, Van Everdingen[6] described gross alterations in structure of starch and glycogen molecules and, more recently, Bach, Luzzio, and Brownell[1] reported alterations in the electrophoretic pattern and antigenic activity of human gamma globulin following exposure to rf energy. These results must, however, be regarded as suggestive rather than definitive at the present time. Similarly, Teixeira-Pinto and colleagues[5] have reported alterations in motility phenomena in unicellular organisms but did not measure the temperature during irradiation.

B. Microwave Equipment and Instrumentation.

1. Electronic Equipment - Plans have recently been instituted to modify some of the existing equipment in order to obtain higher field-strengths with low power output. These plans include:

a. Modification of the present IFI amplifier by means of a special impedance matching circuit originally designed by General Telephone Co.

b. Installation of a digital read out frequency meter to modify the Hewlett-Packard oscillator in order to provide constant monitoring of frequency.

c. Installation of a copper screen room in the laboratory in order to prevent interference with other electrical equipment in the building. The room will be provided with a good grounding connection which is said to be very important with respect to the reproducibility of the results.

2. Irradiation Chambers - We have established a small tissue culture laboratory which is presently in operation. It was necessary to acquire a small amount of basic equipment and some time was spent reviewing and learning technics for in vitro culture of mammalian cells. A brief review of the literature has revealed that little has been done with microwave exposure of tissue cultures to the present time. The work with unicellular organisms will be helpful in establishing general parameters of frequency, field strength, and temperature control for these exposures, and we have technics of biochemistry, histochemistry and electron microscopy at our disposal which will facilitate study and documentation of any effects observed directly.

C. Studies on Macromolecules.

1. Non-thermal Effects -

a. Bovine serum albumin solutions were irradiated by a pulsed radio frequency energy at 1, 2, 5, 10, 20, 30, 45, 50, and 60 mc. The field intensity was 100 and 500 volts and the pulse duration was 10 microseconds with a 2 millisecond interval. The temperature was

controlled by circulating thermostated water and the temperature was recorded by inserting a copper-constantan thermocouple immediately after irradiation. No temperature rise was observed with these field intensities. After irradiation, the protein solutions were placed in a Sephadex - 50 column which has been shown to separate denatured from native serum albumin. It was, however, found that the rf field did not produce any measurable change in the column pattern. Increase of field intensity, prolonged irradiation and irradiation at different pH values did not produce any detectable change. It was, therefore, concluded that radio-frequency energy at these frequencies has no non-thermal effect on the structure of bovine albumin.

b. Another protein, yeast alcohol dehydrogenase, was irradiated and assayed for enzymatic activity. The frequencies utilized ranged from 1 to 10 mc and the field intensity was 100 and 300 volts across the solution; the duty cycle was originally 1% but was increased to 40% which was the maximum duty cycle which could be applied to the buffered solution without heating. The temperature was strictly controlled by circulating ice cold water. The activity of alcohol dehydrogenase was assayed by reduction of diphosphopyridine nucleotide which was measured at 340 mμ in a Beckman DU spectrophotometer. As this reaction is a first order one, a logarithmic plot of velocity against time is linear. The linear portion of this plot was used for comparison of enzyme activities in irradiated and non-irradiated samples. No change

in activity was noted at any of the frequencies studied; alteration in activity was only observed when the temperature was allowed to rise.

c. Studies on DNA - Solutions of DNA were initially irradiated at frequencies from 1 - 10 mc at a field intensity of 200 volts. Measurement of vicosity was used to detect alterations in the structure of DNA. It was found, however, that irradiation at this frequency was without effect on DNA.

Since it was previously shown by Takashima[4] that the dielectric absorption of electromagnetic energy by DNA is maximal at 500 c, measurements were made in this region using a square wave generator at frequencies ranging from 20c to 5 kc, at a field intensity of 5 volts. Solutions were irradiated for 30 minutes and the viscosity was measured with a conventional Ostwald viscosimeter. A marked effect on the viscosity of DNA solutions was observed, which was maximal at 500 c. Electron microscopic studies were carried out on the irradiated DNA and it was found that square wave fields indeed ruptured the DNA chains and produced very small fragments. The effect of the square wave field, however, can be due to the d.c. component as well as to the a.c. component since square wave generators always put out d.c. field too. To test the significance of this, we set up an irradiation system which consists of General Radio sine wave oscillator and a shielded transformer which amplified the intensity four times. The peak to peak intensity across the DNA solution was 150 volts and no d.c. component was detected. The irradiation was

carried out between 10 c and 10 kc and the change in the structure of DNA was examined by measuring the viscosity of the solution as before. In contrast to the square wave field, the sine wave electromagnetic field did not alter the viscosity of the DNA solution even after prolonged irradiation. The results were consistently negative. Therefore, we had to conclude that the effect which had been observed with square wave field had been due merely to the electrolysis effect of the d.c. component.

2. Thermal Effects (See Fig. 1) -

The heat generation by the microwave is the great disadvantage for the study of non-thermal effect. This advantage, however, can be a great advantage for another purpose. High power radio waves can be used for the step heating of a solution and initiate a thermally induced reaction. The thermal denaturation of DNA has been studied rigorously by many people but the kinetics have not been studied because of the difficulty of maintaining the high temperature. We found that the field at 100 watts increased the temperature of DNA solution from 25°C to 100°C in 30 seconds, and also that the high temperature could be maintained for a long period of time. Platinum electrodes were inserted in the optical density measuring cell and the thermal denaturation was followed by recording the increase of the optical density at 260 Å which is the characteristic absorption of nucleic acids. (Fig. 1) We found that the thermal denaturation was completed in 15 seconds. This is much longer than the predicted

theoretical values given by Longuett-Higgins and Zimm[8] (1.4 sec) and Fixman[9] (0.2 sec). More refinement of the instruments is under progress and more reliable data will be produced.

D. Effects on Motility of Unicellular Organisms.

In the period of time since the last report the only progress in this area has been the further confirmation of the orientation of the movements of ciliates such as Paramecium. In addition to orientation of swimming movements, certain organisms have been observed to either spin about a central axis or to precess around one pole. This effect is clearly related to the electromagnetic field as the rate of rotation is directly proportional to the field intensity. We are presently able to produce orientation of these organisms in a direction perpendicular to the electrodes; it is hoped that with increase in the field strength made possible by the equipment modifications described above, that the orientation parallel with the electrodes, reported by Heller, can be produced.

E. Effects on Tissue Culture.

Some definite progress has been made in this portion of the project by the development of an improved cell for subjecting cultures to microwave radiation, but due to difficulties in establishing a hardy cell line work has not progressed. The cells obtained for culture (L strain fibroblasts, Flow Laboratories, Rockville, Maryland) did not survive transfer from the original culture flask, and after several attempts it has been decided to obtain new cultures.

The cell that has been devised for subjecting the cultures to microwave (Fig.2) consists of an outer tube of bronze connected to one electrode, and an inner core of solid bronze constituting the other electrode, which is coupled to an electric motor. The glass culture flask can be placed in tubular chamber so that the central bar projects into the center of the flask, and the entire assembly can be rotated by the electric motor to maintain the cells in suspension. The irradiation chamber has been constructed by the Biophysics Branch of the Armed Forces Institute of Pathology, but has not been tested as yet because of the difficulty with the cell strain.

F. Other Activities.

1. During December 1962, Dr. Takashima visited the laboratory of Dr. Sven Bach at the U.S.Army Medical Research laboratory, Fort Knox, Kentucky. He discussed the work of that group in relation to the results obtained in our laboratory.

2. During May, we made a one-day trip to the New England Institute for Medical Research, under the direction of Dr. John Heller, to discuss the results which they had obtained. The effect of radio frequency wave on mitosis particularly interested us. They observed various kinds of deformation of the chromosomes after the irradiation. We also found that the most fundamental difference between their instrumentations and ours is in the field intensity. They are using a field intensity of 2000 or more volts, peak to peak, while we use only 200 volts, peak to peak. Dr. Heller claimed that no effect

would be observed at low field intensity and recommended that the field intensity of our instruments be increased. We have already started the remodeling which will be completed in a few months.

2. The effects of audio frequency waves on DNA were reported orally at the meeting of the Federation of American Societies for Experimental Biology at Atlantic City, in April, 1963. (Fed. Proc. 22: 670, 1963)

G. Future Plans.

Future experiments on this project are being carried out along the following lines:

1. Remodeling the instruments to increase the field intensity. The following studies will all be done with the modified instrument.

2. Studies of cell division chromosomes and mitosis.

3. Studies on the structure of DNA.

4. Studies on enzyme activity, particularly yeast alcohol dehydrogenase.

5. Studies on the thermal denaturation of DNA. This is to study the kinetics of uncoiling of DNA.

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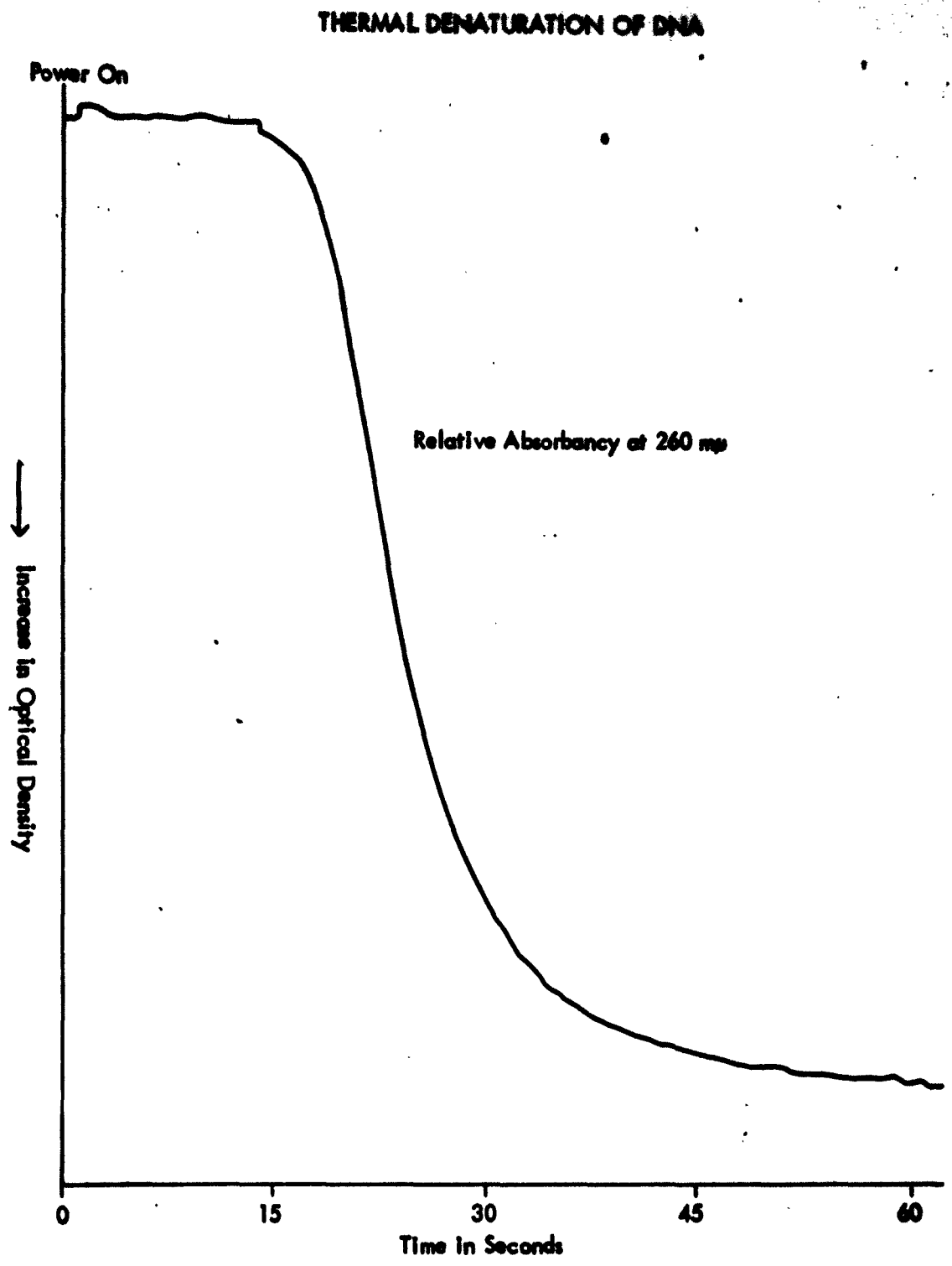
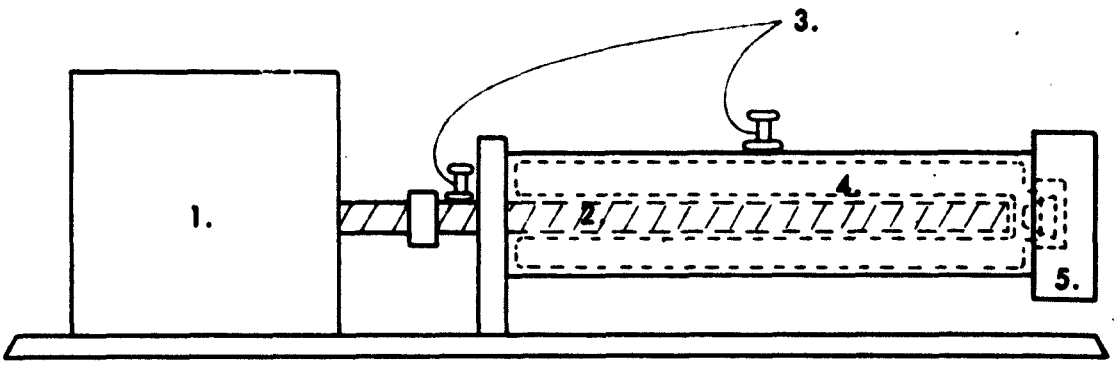

THERMAL DENATURATION OF DNA
Power On
Relative Absorbancy at 260 mμ
Increase in Optical Density
0
15
30
45
60
Time in Seconds

Figure 1.

MICROWAVE CELL FOR IRRADIATION OF TISSUE CULTURE



1. Electric motor
2. Core electrode
3. Electrode connectors to amplifier
4. Glass vial containing tissue culture
5. Polyethylene cap

Figure 2

ANNUAL PROGRESS REPORT

Title Page

Project No. 3A012501A803

Task No. 12 **"Studies on Toxic Agents Obtained by Oxidation of Highly Unsaturated Fatty Acids"**

Name & Address of Reportion Installation:

Armed Forces Institute of Pathology

Washington 25, D. C.

Name of Department(s) and Division(s):

Department of Pathology

Division of Basic Sciences

Biochemistry Branch

Period Covered by the Report: 1 July 1962-30 June 1963

Professional Authors of the Report:

Principal Investigator: V. S. Waravdekar, Ph.D.

Assistant: L. D. Saslaw, Ph.D.

Reports Control Symbol (RCS-MEDDH-288)

Security Classification: (Unclassified)

ABSTRACT

Project No. 3A012501A803 Title Internal Medicine

Task No. 12 Title Studies on Toxic Agents Obtained by Oxidation of Highly Unsaturated Fatty Acids

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology

Washington 25, D. C.

Period Covered by the Report:

1 July 1962 - 30 June 1963

Authors: V. S. Waravdekar, Ph.D.

L. D. Saslaw, Ph.D.

Reports Control Symbol: (RCS MEDDH-288)

Security Classification: Unclassified

SUMMARY

Two thiobarbituric acid-active components were detected in the aqueous extracts prepared after ultraviolet irradiation of polyenoic fatty acids. One of the components exhibits an absorption maximum at 225 mμ. Both components are associated with material which is inhibitory to several mouse liver enzymes and the extracts which contain both activities were found to be lethal to mice and to produce abnormal changes in mouse liver.

BODY OF REPORT

Project No. 3A012501A803 Title Internal Medicine

Task No. 12 Title Studies on Toxic Agents Obtained by Oxidation of Highly Unsaturated Fatty Acids

Description:

The primary objective of this project is to investigate the nature of compounds obtained by oxidation of naturally-occurring highly unsaturated fatty acids and to study their effects on mammalian systems. It is proposed to obtain information of importance to military medicine concerning the oxidation of these fatty acids as induced by radiation and by chemical oxidizing agents.

Specific Aims of the Project: (a) To obtain information on the mechanisms involved in the fatty acid oxidation (b) To obtain basic knowledge on the mode of action of the toxic agents on cellular systems, and (c) To investigate the role of antioxidants in controlling and preventing fatty acid oxidation.

Progress:

Ultraviolet radiation has been employed to provoke oxidation of polyenoic fatty acids and esters. Aqueous extracts prepared from the irradiated lipids have been found to be lethal to mice upon I. P. administration. Administration of small quantities of extract resulted in liver damage as was evidenced by enlargement and a suggestive proliferative appearance. Studies are being continued on the toxic effects.

In in vitro studies, the aqueous extracts were found to inhibit a number of mouse liver enzymes in proportion to the extent of oxidation of the lipids. The extent of oxidation was followed principally by reference to the thiobarbituric acid assay. When the aqueous extracts were further fractionated by washing with organic solvents, it was found that practically no thiobarbitruic-acid active material was removed. However, most of the material which was inhibitory to enzymes was removed by solvent washing.

The remaining aqueous phase was studied as to the nature of the thiobarbituric acid-active constituents. By the use of countercurrent distribution techniques, it was found that two thiobarbituric acid-active components were present. The component which was responsible for the major portion of the thiobarbituric acid activity also exhibited pronounced absorption in the ultraviolet region at 225 mμ which was the predominant ultraviolet spectral feature of the extracts. Based on the ultraviolet absorption spectra of the extracts, no evidence for the presence of peroxides of the fatty acids was obtained.

The same aqueous phase (after solvent washings) was also fractionated by the use of thin-layer chromatography. Two pink thiobarbituric acid-active components and one yellow component were detected. Again, the major portion of the thiobarbituric acid activity and the ultraviolet activity were found to be due to the presence of one component.

In each of the fractionation procedures, the pink thiobarbituric acid-active components were indistinguishable from malonaldehyde on the basis of the visible absorption spectra obtained. No evidence for the presence of malonaldehyde was obtained.

Studies are in progress on the nature of the toxic components and the thiobarbituric acid-active components.

Summary and Conclusions:

Ultraviolet irradiation of polyenoic fatty acids causes the production of two thiobarbituric acid-active components which are indistinguishable from malonaldehyde in the thiobarbituric acid test. No malonaldehyde was produced by the irradiation process. The extracts containing the thiobarbituric acid activity were found to be lethal to mice upon I. P. injection and caused abnormal changes in the liver. The extracts also inhibited a number of mouse liver enzymes, but the inhibition is primarily due to products other than the thiobarbituric acid-active products.

List of Publications:

Ultraviolet Photolysis of Unsaturated Fatty Acids, Fed. Proc. 22, 199 (1963).

Ultraviolet Photolysis of Unsaturated Fatty Acids in Relation to the Thiobarbituric Acid Test, (Submitted to Nature).

ANNUAL PROGRESS REPORT

Title Page

Project No. 3A012501A806 Quantitative Cytology with the Electron Microscope

Task No. 13 Application to Biological Objects

Name and address of Reporting Installation:

Armed Forces Institute of Pathology

Washington, D. C.

Name of Department and Division:

Department of Pathology

Division of Basic Sciences

Biophysics Branch

Period Covered by the Report: 1 July 1961 - 30 June 1963

Professional Authors of the Report:

Principal Investigator: G. F. Bahr, M. D.

Assistant: Elmar H. Zeitler, Ph.D.

Reports Control Symbol: (RCS-MEDDH-288)

Security Classification: (Unclassified)

ABSTRACT

Project No. 3A012501A806 **Title: Quantitative Cytology with the Electron Microscope**

Task: Application to Biological Objects (13)

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology

Washington, D. C.

Period Covered by the Report:

1 July 1962 - 30 June 1963

Authors: G. F. Bahr, M. D. and E. H. Zeitler, Ph.D.

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SUMMARY

The purpose of this research project is to provide the Electron Microscopist with methods for the determination of the mass and composition of small biological objects well beyond the range of any other technique for mass (dry weight) determination.

In 1961-1962 we successfully reported about the first applications of Quantitative Electron Microscopy. The year of this report, 1962-1963, was devoted to demonstrating the wide range of application of Quantitative Electron Microscopy:

A. The weight distribution of human erythrocytes were determined. A distinct difference between normal and microcytic samples was found.

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Task: Application to Biological Objects

B. A study of bull spermatozoa was performed utilizing morphologic and Quantitative Electron Microscopy.

C. A study was undertaken to determine the presence of a weight distribution in a virus population (vaccinia virus).

BODY OF REPORT

Project No. 3A012501A806 Quantitative Cytology with the Electron Microscope

A. Determination of the Total Dry Mass of Human Erythrocytes by Quantitative Electron Microscopy

The dry mass of human erythrocytes has recently been determined by Gamble and Glick (1) in order to compare the values obtained by interference microscopy with those of microradiography. A very good agreement was found.

This study compares the values obtained for the total dry mass of individual human erythrocytes by the two aforementioned techniques and by Quantitative Electron Microscopy. It was hoped to introduce a useful technique in hematology and a new parameter, the dry mass of individual erythrocytes, as aids in clinical diagnosis. We also established the upper limit of practical applicability of Quantitative Electron Microscopy.

Interference microscopy must take into account the optical absorbance of the object, its shape, or both, whereas microradiography requires knowledge of the mass absorption coefficient. The great advantage of the electron microscopic procedures is its independence of shape, optical absorbance, and chemical composition. No a priori knowledge as to the composition or shape of the object is required. Only one assumption must be made--that object and standard behave comparably during exposure in the electron microscope.

The total dry mass of each of 1,800 human erythrocytes from seven healthy persons was determined by Quantitative Electron Microscopy. The median weight of an erythrocyte was found to be 29.9×10^{-12} gm., with a range of 28.1×10^{-12} gm. to 32.0×10^{-12} gm. These values are in close agreement with those obtained by interference microscopy or microradiography.

The weight of erythrocytes follows a logarithmic normal distribution; there is a remarkable uniformity of the data from different persons. A comparison with the distribution of erythrocyte diameters (Price-Jones curve) is given.

The results of this study demonstrate the applicability of Quantitative Electron Microscopy to biologic objects weighing up to 5×10^{-11} gm. This technique overlaps the lower limits of interference microscopy and microradiography.

Quantitative Electron Microscopy was applied to the study of erythrocytes in microcytic anemia and the results are shown in Fig. 1.

B. Study of Bull Spermatozoa

The electron microscope has been used to determine the characteristic dimensions and the distribution of the dry mass in bull spermatozoa. Morphologic observations on whole mounted and trypsin- or pepsin-treated preparations support the quantitative findings.

The present study clearly demonstrates a lack of correlation between the mass and length of major sperm structures. It is felt that this finding carries much biologic significance. Further investigation is needed to determine whether this principle of "lack of correlation" is true only of sperm or whether it constitutes a more general principle applicable to the nuclear mass and other parameters of ordinary cells.

A particularly interesting finding of this study is the fact that spermatozoa of the bull, the heads as well as the tails, their masses as well as their geometric parameters, are distributed in distinct populations. This finding is in keeping with the earlier results of Quantitative Electron Microscopy on mass distribution in biologic populations (2). Most noteworthy is the fact that these distributions are not normal but logarithmic-normal.

The occurrence in logarithmic distributions as well as the non-correlation of parts in the assembly of a spermatozoon are considered to reflect significant biologic principles.

Methodologically, a new procedure is added to Quantitative Electron Microscopy permitting the recording of the mass cross section (total mass per unit length) of an object. This approach makes possible determinations of the distribution and the total mass of very long and narrow structures. Fig. 2 shows a mass profile of a sperm derived from such a scan.

C. Weight Distribution of Vaccinia Virus

Because the mass of viruses lie at the lower level of applicability of Quantitative Electron Microscopy, they were selected for study. Viruses are further interesting in that their simple structure allows for measuring variations in mass induced by digestion or staining. Vaccinia, a large virus, was choosen as a starting point. The median weight determined from 800 individual measurements was 5.9×10^{-15} gms. The spread of the distribution found is distinct from that of the errors of measurement. The values obtained are slightly larger than those derived from ultra-centrifugation and other indirect techniques. It should be pointed out that these techniques cannot provide critical data on distribution.

References:

(1) Gamble, C. N. and Glick, D., J. Cell Biol. 8, 53, 1960.

(2) Bahr, G. F. and Zeitler, E., J. Cell Biol. 15, 489, 1962.

Reports:

Reports on this research were presented to 1) Roswell Park Memorial Hospital, Buffalo, New York, July 1962, 2) University of Minnesota, Minneapolis, Minnesota, July 1962, 3) Fifth International Conference for Electron Microscopy, Philadelphia, Pennsylvania, August 1962, 4) American Society for Cell Biology, San Francisco, California, November 1962, 5) American Society of Hematology, Columbus, Ohio, November 1962, 6) American Academy of Oral Pathology, Miami, Florida, April 1963.

Project No. 3A012501A806

Publications:

1. Bahr, G. F. and Zeitler, E., Lab. Invest. 11, 912-917, 1962.

2. Zeitler, E. and Bahr, G. F., RCA Scientific Instruments News 7, 3-11, 1962.

3. Bahr, G. F. and Zeitler, E., J. Cell Biol. 15, 489, 1962.

Legends

Figure 1

Weight distribution of human erythrocytes. Crosses: normal blood; open circles: microcytic anemia. F = integral frequency; W = weight.

Figure 2

Example of a recording of the weight per length (W/L) from a longitudinal scan over the entire sperm with a slotted aperture. Simultaneously the total weight (W) as a function of the length (L) is shown. The intersection of the integral weight curve with the 50 per cent line of the W coordinate indicates the location of the center of gravity. At about 23 μ a slight "elevation" marks the annulus and at about 28 μ a "dip" marks the termination of the first pair of the outer fibers.

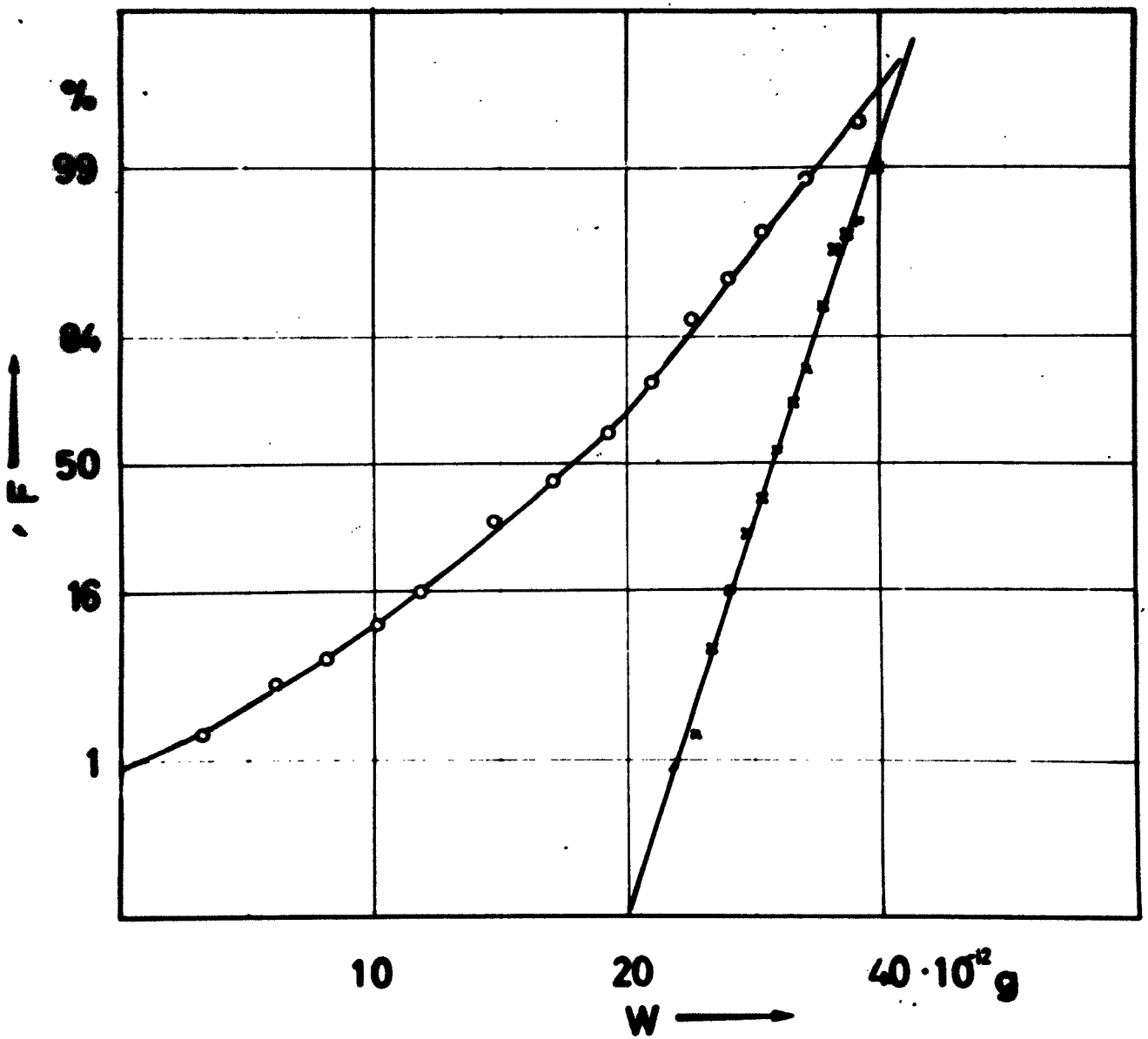

Fig. 1

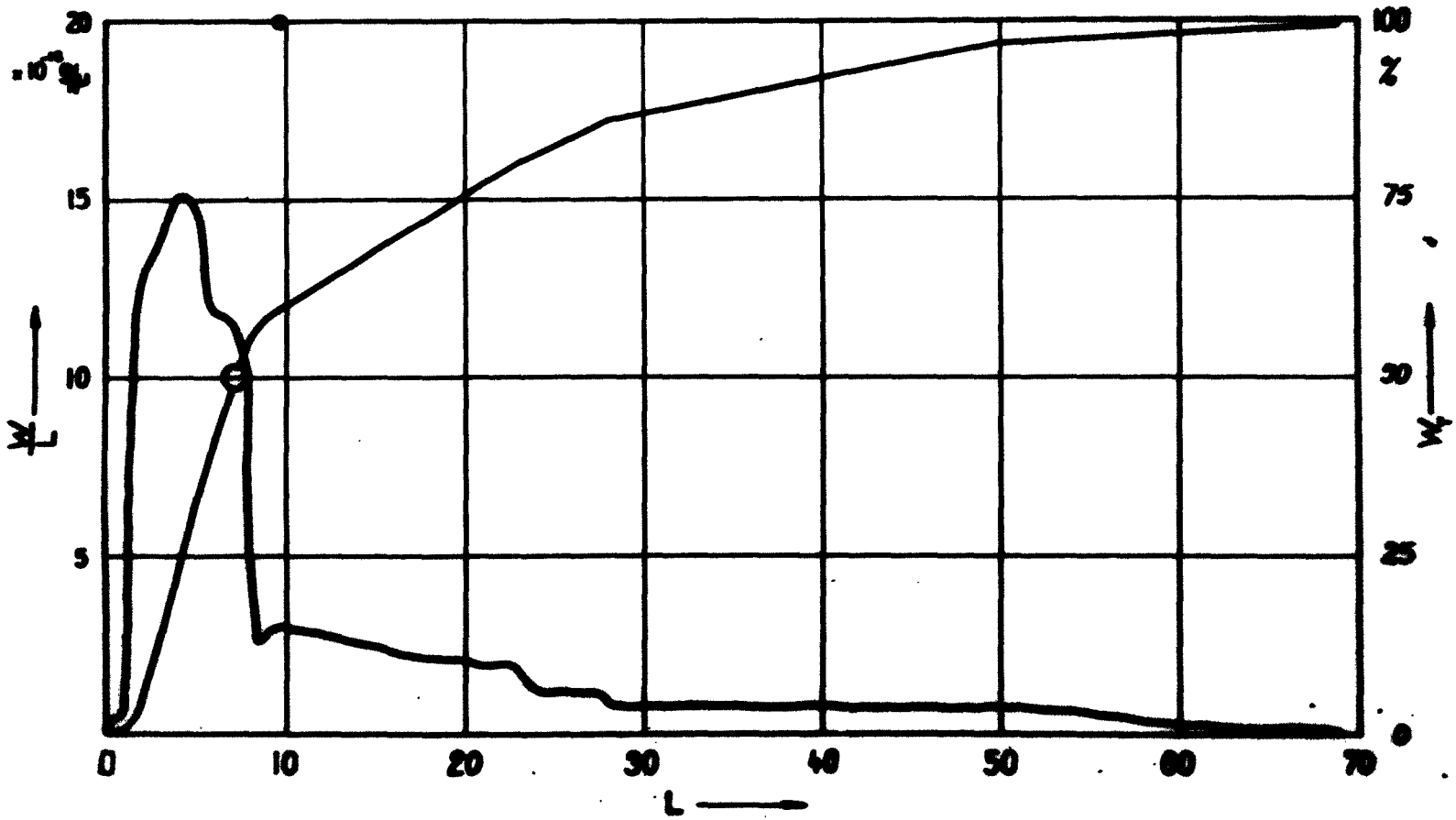

Fig. 2

ANNUAL PROGRESS REPORT

PROJECT TITLE: 3A012501A802 Traumatic Surgery and Shock

TASK: 14 Structure and Function of Ocular Tissue (No. 8)

REPORTING INSTALLATION: Armed Forces Institute of Pathology
Washington 25, D. C.

DEPARTMENT AND DIVISION: Department of Pathology
Division B-General and Special Pathology
Ophthalmic Pathology Branch

PERIOD COVERED: 1 July 1962 - 30 June 1963

AUTHORS:

PRINCIPAL INVESTIGATORS: Benjamin Rones, M. D., and
Lorenz E. Zimmerman, M. D.

ASSISTANT: Ben S. Fine, M. D.

REPORTS CONTROL SYMBOL: (RCS-MEDDH-288)

SECURITY CLASSIFICATION: Unclassified

ABSTRACT

PROJECT TITLE: 3A012501A802 Traumatic Survery and Shock

TASK: 14 Structure and Function of Ocular Tissue

REPORTING INSTALLATION: Armed Forces Institute of Pathology
Washington 25, D. C.

PERIOD COVERED: L July 1962 - 30 June 1963

AUTHORS: Benjamin Rones, M. D., and Lorenz E. Zimmerman, M. D.

ASSISTANT: Ber. S. Fine, M. D.

REPORTS CONTROL SYMBOL: (RCS-MEDDH-288)

This progress report covers the second full year of support since activation of this project on 1 September 1960. During this period further study has been made on the human retina by light and electron microscopy. The ultrastructure of the four previously known "limiting membranes" of the retina was determined[1] and from a synthesis of the information derived from these studies and light microscopic observations of both normal and pathologic retina, a fifth or "middle limiting membrane" of the human retina was established.[2]

Similarly, observations on the synaptic regions of the rods and cones were applied to a study of the inner plexiform layer where synapses are to be found in abundance, and similar synaptic arrangements were found "in miniature."[3] This provides an additional criterion for future identification of certain synaptic regions in the human retina and possibly elsewhere in the central nervous system.

The third neuronal level or ganglion cell layer was investigated from both peripheral (nasal) and macular (paracentral) regions

of human retina, with particular reference to the presence of dense intracellular granules which are considered to be the anatomic basis for the clinically observable yellow color of the macula lutea.[4] A study of the photoreceptor outer and inner segments was completed[5] with particular reference to the presence of an extracellular mucinous material within the spaces between these outer segments. Evidence was presented which indicates that the inner segments of the photoreceptor cell is the likeliest source for elaboration of much of this material.

An extensive study has been completed and published[6] on the nonpigmented epithelium of the pars plana region of the ciliary body in both man and rhesus monkey. Morphologic differences which have long been known but generally not appreciated between cells near the ora serrata and the anterior pars plana were re-examined. Anatomic evidence was obtained which helped explain the strong attachment of the vitreous body in this region. Combining techniques of light microscopy, histochemistry and electron microscopy, evidence was obtained that much of the intraocular mucinous material (mainly hyaluronic acid) is synthesized by these nonpigmented ciliary epithelial cells. These observations were also applied to the study of a tumor derived from the nonpigmented ciliary epithelium, proving of value in understanding some of the peculiarities of this tumor.

Further observations were made on normal lens material[7] as well as cataractous lenses. The opportunity presented itself for us to study a case of triparanol (MER-29) cataract.

Further observations on the human retina will be made during the coming year. A study of the normal rabbit retina will be initiated in order to obtain background for the study of experimentally produced photocoagulation burns of the retina in this animal, in collaboration with Drs. Geeraets and Ham at the Medical College of Virginia in Richmond.

DESCRIPTION

Research during this period has continued into the structure of the normal human retina. For better appreciation of some of the anatomic questions involved in retinal structure, an extensive examination was made on the nonpigmented cell layer of the ciliary pars plana, a single cell layer extension of the sensory retina. Detailed investigation of this neuroepithelial layer revealed that much of the intraocular mucinous material is synthesized by these cells in the adult eye. Anatomic details of the complex interweaving of multiple layers of fenestrated basement membrane, cell villi and flaps, and vitreous filaments went far to explain the strong attachment of the vitreous body across the region of the ora serrata (vitreous "base"). Sites of probable intracellular synthesis of these proteinaceous and mucinous materials were observed, and a pathway for their passage into the ocular cavities was proposed. The nonpigmented cells elaborate their products from the apical cytoplasm (as do most other mucus-secreting cells). These pars plana cells, being inverted during

embryologic development, and their apical ends being tightly bound to an adjacent layer of pigmented cells, must secrete their materials laterally near their embryologic apexes into intercellular spaces. Passing through these tortuous intercellular spaces the material then makes its way through the basement membrane of the nonpigmented ciliary epithelium into the aqueous and vitreous.

Application of these observations to a study of a well differentiated malignant tumor of the nonpigmented ciliary epithelium showed a striking morphologic similarity of the locally invasive tumor cells to the normal epithelial cells. Abnormal quantities of basement membrane were, however, observed to be produced by the tumor cells.[8] Further observations were made on the sensory retina with attention directed toward the substance occupying the spaces between the photoreceptor outer segments.[5] This material has been described previously as a mucinous material, and has been shown to contain a hyaluronidase-resistant acid mucopolysaccharide. Because of the presence of pigment epithelial cell processes between these photoreceptor cells (esp. in certain amphibia) and the limits of resolution of the light microscope, question has been raised in some quarters as to whether this material is intracellular, extracellular or both.

Combining techniques of light and electron microscopy in a study of human photoreceptors the mucinous material was found to be almost completely extracellular. Further observations indicated that a primary source for elaboration of this material was in the rod and cone inner segments.

A study of the ganglion cells of the retina has been published. This study of the electron microscopic appearance of these large neurons was concerned mainly with their content of electron dense granules. These ganglion cell granules apparently increase in both number and density with age. It became apparent that these granules were the probable anatomic basis for the yellow coloration of the macula lutea, where the ganglion cells are found in sufficient concentration to produce a yellow color, easily observed in the enucleated eye or in a high percentage of patients when they are examined with a light of suitable wavelengths (i.e. white light). This yellow region immediately surrounds the fovea centralis, the critical region of greatest visual acuity which itself is devoid of both ganglion cells and pigmentation.

Further observations have been made on the normal human lens as a basis for further study of pathologic material (i. e. disease produced cataracts, drug induced cataracts or cataracts produced by various forms of electromagnetic radiation).

The anterior and posterior lens capsules, together with the adherent zonular fibers and vitreous body were examined to review their normal relationships. The epithelium and its relationship to the underlying cortex was studied as well as the differences between cells of the superficial and deep lens cortex. Human cataracts produced by long term therapy with triparanol (MER-29) have become available and studies are in progress on this material. This drug

appears to produce widespread changes in the lens cortex and epithelium which seem to differ somewhat from the changes generally associated with exposure to electromagnetic or ionizing forms of radiation. The observations on the normal human lens have been contributed towards a textbook on the biochemistry of the lens by Dr. Sidney Lerman.

FURTHER STUDIES

Investigation will continue into the structure of the normal human retina. Although some studies can be made on the effect of photocoagulation on the human retina in certain selected volunteers (e.g. where the eye is to be enucleated for a well localized tumor) it is now felt that a correlated study should be initiated on the normal rabbit retina since considerable equipment and information has been developed in the past several years to utilize this readily available and inexpensive animal for these studies. Information available at present indicates that there is sufficiently small difference between the effect of photocoagulation on the rabbit retina and extramacular regions of the human retina, so that extrapolation of information from one species to the other should prove significant.

With knowledge gained in the study of the normal rabbit retina, photocoagulation burns (which are considered similar to, but more reproducible than those produced by laser sources) of the rabbit retina will be examined.

Further application of information obtained from the study of normal tissues to the study of pathologic material will be made from time to time as this latter material becomes available. The acquired information will then be useful in interpreting experimentally produced lesions.

Training of a full time Fellow in ophthalmic pathology in the techniques and interpretations of electron microscopy did not materialize as expected. A part time trainee ophthalmologist has recently arrived, and his training in electron microscopy has begun. Study has commenced on the structure of the normal cat cornea as a preliminary to future experimental work. During this initial training period the trainee is receiving instruction in fixation, embedding and sectioning of corneal tissue, examination and photography by electron microscopy and interpretation of the micrographs so obtained.

An extensive bibliography of the pertinent literature is available to the trainee. Already he has obtained a few satisfactory micrographs of normal cat cornea.

SUMMARY AND CONCLUSIONS

1. Detailed knowledge of normal structure is essential in preparation for any further investigation of ocular pathology or experimental ocular lesions.

2. Considerable study has been made on human lens, iris, retin vitreous body and the ciliary epithelium.

3. A study of the rod and cone layers of the human retina has been completed and is ready to be submitted for publication.

4. Some observations of the normal ciliary epithelium have been applied to study of a locally invasive tumor of this cell layer. A predominantly light microscopic study of these tumors is in preparation.

5. A study of the normal rabbit retina is to be initiated to correlate with the known observations both human, and those already published of the rabbit, as a preparation for the study of experimental photocoagulation lesions of the rabbit retina.

6. A part time trainee has arrived, is receiving instruction, and has commenced a study of the normal cat cornea as a preliminary to the study of experimental lesions.

7. A list of publications already published, in press, completed or in preparation is appended.

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9. Fine, B. S.: Letter to the editor and micrographs in clarification of certain points of confused anatomy. Arch. Ophthal. 67: 689, 1962.

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